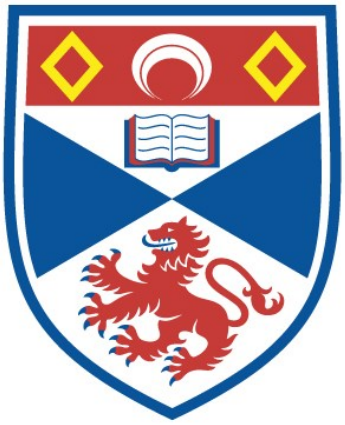
INTRINSIC AND EXTRINSIC FACTORS INVOLVED IN THE AXONAL GROWTH RATE OF EMBRYONIC NEURONS

Mark Christopher Hilton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1999

INTRINSIC AND EXTRINSIC FACTORS INVOLVED IN THE AXONAL GROWTH RATE OF EMBRYONIC NEURONS

A thesis submitted to the University of St. Andrews

for the degree of Doctor of Philosophy (Ph.D.)

by

Mark Christopher Hilton

July, 1999.

School of Biomedical Sciences

Bute Medical Building

St.Andrews

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Ta ynsagh coamrey stoamey yn dooinney berchagh;

as t'ec berchys yn dooinney boght

Ny insh dou ny mee agh insh dou ny va mee

I dedicate this thesis to my parents, for their love and patience

Declarations

I, Mark Christopher Hilton, hereby certify that this thesis, which is approximately 58729 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 26/8/99 Signature of candidate

I was submitted as a research student in October, 1996 and as a candidate for the degree of PhD in October 1996; the higher study for which this is a record was carried out in the University of St. Andrews between 1996 and 1999.

Date 26/8/99 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application of the degree.

26/8/99

Date Signature of supervisor

Copyright

Acknowledgments

I would like to express my most sincere thanks to my supervisor, Professor Alun Millward Davies for giving me the opportunity to undertake this project in his laboratory and without whose time, patience and understanding this thesis would not have been possible.

I would like to thank my long suffering office companions Jane Thompson, Fleur Davey, Oliver Schmidt and Rosa Andres for making my time spent in the lab three very enjoyable years that I will always look back on with happiness, and for their friendship which I will always cherish.

I would like express my sincere gratitude to Sean Wyatt and Gayle Middleton for reading this thesis in such a short time and for their help and advice that they gave me during the writing of this thesis.

I am grateful to Flavio Maina for collaborating in chapters 3 and 4, Fleur Davey for collaborating in chapter 5 and Gayle Middleton for collaborating in chapter 6

Thanks to all my friends and colleagues in the lab past and present Alison Forgie, Luc'a Pinon, Rosa Soler, Tony Horton, Jimi Adu, Vladimir Buchman, Natalia Ninkina, Liz Delaney, Ruth Edgar, Katrin Huber and Epaminondas Doxakis.

Table of Contents

Abstract

The main aim of this project has been to study the extrinsic and intrinsic factors involved in the axonal growth rate of sensory and sympathetic neurons during the early stages of their development. Using *in vitro* assays it was shown that Hepatocyte Growth Factor (HGF)/Met signalling significantly enhanced the survival and neurite length of early DRG neurons grown in the presence of NGF. This synergism was specific for NGF but not for the related neurotrophins BDNF or NT-3 (Maina et al., 1997). HGF/Met signalling was also shown to accelerate the differentiation of sympathetic neurons as well as promoting the survival of sympathetic neuroblasts but not postmitotic neurons. HGF was also shown to cooperate with NGF to enhance the axonal growth rate as well as increasing the amount of neurite branching of NGF-dependent sympathetic neurons throughout development (Maina et al., 1998). HGF was also shown to enhance the survival and growth of developing parasympathetic and proprioceptive neurons. The demonstration that HGF only enhanced the survival and growth of proprioceptive TMN neurons when grown in CNTF but not when grown in BDNF which promoted their survival as effectively as CNTF demonstrates that within the same neurons, the effects of HGF on survival and growth are selectively dependent on which other signalling pathways are concurrently activated. The anti-apoptotic protein Bcl-2 has been shown to play a key role in regulating cell survival in the nervous system. Cultured neurons expressing antisense Bcl-2 RNA have an attenuated survival response to neurotrophins, and neurons of postnatal Bcl-2 deficient mice die more rapidly following NGF deprivation *in vitro* and are present in reduced numbers in vivo. Here I show that Bcl-2 also plays a key role in regulating axonal outgrowth rates in embryonic neurons (Hilton et al., 1997). The effect of Bcl-2 on axonal growth rate

was shown not to be a consequence of its well documented role in preventing apoptosis.

List of Figures

Chapter 1

Chapter 2

Chapter 3

Figure 3.3. Bar chart showing the percent survival of E12 DRG neurons cultured in BDNF (5 ng/ml) or plus HGF (5 ng/ml).

Figure 3.4. Bar chart showing the percent survival of E12 DRG neurons cultured in NT-3 (5ng/ml) or plus HGF (5ng/ml).

Figure 3.5. Graph showing neurite length of E12 DRG neurons cultured in NGF (5ng/ml) alone or NGF plus HGF (5ng/ml).

Figure 3.6. Camera lucida drawings showing typical appearance of E12 DRG neurons cultured in NGF (5ng/ml)alone or plus HGF (5ng/ml).

Chapter 4

Figure 4.1. Photomicrograph showing representative postmitotic sympathetic neurons and sympathetic neuroblasts.

Figure 4.2. Bar chart of E13.5 SCG neuroblasts that differentiate at intervals in culture in control cultures and cultures containing HGF (10ng/ml) or anti-HGF (1.25mg/ml).

Figure 4.3. Bar chart showing percentage of E12.5, E13.5 and E14.5 SCG neuroblasts that differentiate after 24hrs in culture with HGF (10ng/ml) or anti-HGF (1.25mg/ml).

Figure 4.4. Bar chart showing percentage of SCG neuroblasts from wild-type and *met*$^{d/d}$ embryos that differentiate after 24hrs in culture with HGF (10ng/ml) or anti-HGF (1.25mg/ml).

Figure 4.5. Bar chart showing percentage of SCG neuroblasts that die prior to differentiating into neurons in control cultures and cultures containing HGF (10ng/ml) or anti-HGF (1.25mg/ml).

Figure 4.6. Bar chart showing percentage survival of postmitotic sympathetic neurons in cultures containing NGF (2ng/ml) plus HGF (10ng/ml) or NGF plus anti-HGF (1.25mg/ml).

Figure 4.7. Bar chart showing percentage survival of postmitotic sympathetic neurons form wild-type and *met*$^{d/d}$ embryos in cultures containing HGF (10ng/ml) or anti-HGF (1.25mg/ml).

Figure 4.8. **A.** Photomicrographs of cross-sections of SCG from E14.5 and E16 wild-type and *met*$^{d/d}$ embryos. **B.** Bar chart showing quantitative analysis of the number of pyknotic nuclei and neuron size in SCG and nodose ganglia derived from E14.5 wild-type and *met*$^{d/d}$ embryos.

Figure 4.9. Bar chart showing neurite length of SCG neurons at different ages cultured in NGF 2ng/ml alone or plus HGF 10ng/ml or anti-HGF 1.25mg/ml.

Figure 4.10. Bar chart showing neurite length of TPG neurons at different ages cultured in NGF 2ng/ml alone or plus HGF 10ng/ml or anti-HGF 1.25mg/ml.

Figure 4.11. Bar chart of neurite length of E14.5 SCG neurons cultured in NGF 2ng/ml plus HGF 10ng/ml or anti-HGF 1.25mg/ml over 72hrs .

Figure 4.12. Bar chart of neurite length of E14.5 TPG neurons cultured in NGF 2ng/ml plus HGF 10ng/ml or anti-HGF 1.25mg/ml over 72hrs

Figure 4.13. Dose response showing the neurite outgrowth of E14.5 SCG neurons after 48 hrs cultured with a range of HGF concentrations either with or without NGF (2ng/ml).

Figure 4.14. Dose response showing the neurite outgrowth of E14.5 TPG neurons after 48 hrs cultured with a range of HGF concentrations either with or without NGF (2ng/ml).

Figure 4.15. Dose response showing the neurite outgrowth of E14.5 SCG neurons after 48 hrs cultured with a range of NGF concentrations either with or without HGF (10ng/ml).

Chapter 5

Chapter 6

Figure 6.4. Graph comparing the neurite length of E12 trigeminal neurons from Bcl-2 deficient and wildtype embryos cultured in NGF (5ng/ml).

Figure 6.5. Graph comparing the neurite length of E12 trigeminal neurons from Bcl-2 deficient and wildtype embryos cultured in BDNF (5ng/ml).

Figure 6.6. Graph comparing the neurite length of E12 trigeminal neurons From Bcl-2 deficient and wildtype embryos cultured in NT-3 (5ng/ml).

Figure 6.7. Camera lucida drawings showing typical appearance of E11 trigeminal neurons from Bcl-2 deficient and wildtype embryos cultured in 5ng/ml NGF.

Figure 6.8. Camera lucida drawings showing typical appearance of E12 trigeminal neurons from Bcl-2 deficient and wildtype embryos cultured in 5ng/ml NGF.

Abbreviations

Aa Amino Acids

ARTN Artemin

BDNF Brain-derived neurotrophic factor

BrdU Bromodeoxyuridine

BSA Bovine serum albumin

CNS Central nervous system

CNTF Ciliary neurotrophic factor

CT-1 Cardiotropin-1

DMTG Dorsomedial trigeminal ganglia

DRG Dorsal root ganglia

E Embryonic day

EDTA Ethylenediaminetetraacetic acid

F12 Ham nutrient mixture F-12

F14 Ham nutrient mixture F-14

FGF Fibroblast growth factor

GDNF Glial cell line-derived neurotrophic factor

HBSS Hanks buffered salt solution

HGF Hepatocyte growth factor

HIHS Heat-inactivated horse serum

IL-2 Interleukin-2

IL-3 Interleukin-4

IL-5 Interleukin-5

IL-6 Interleukin-6

Kb Kilobase

kDa Kilodaltons

L15 Leibovitz's L15 nutrient mixture

LIF Leukaemia inhibitory factor

mRNA Messenger Ribonucleic Acid

NGF Nerve growth factor

NT-3 Neurotrophin-3

NT-4 Neurotrophin-4

NT-5 Neurotrophin-5

NT-6 Neurotrophin-6

NT-7 Neurotrophin-7

NTN Neurturin

OMS Oncostatin-M

PCR Polymerase chain reaction

PSP Persephin

P-ORN Poly-DL-ornitine

PNS Peripheral nervous system

RPTP Receptor protein tyrosine phospahatase

RT-PCR Reverse Transcription-polymerase chain reaction

RTK Receptor tyrosine kinase

SCG Superior cervical ganglia

SH2 Src homology 2

TAE buffer Tris-acetate-EDTA buffer

TGF-β Transforming growth factor-β

TMN Trigeminal mesencephalic nucleus

TPG Thoracic paravertebral ganglia

VLTG Ventrolateral trigeminal ganglia

CHAPTER 1
GENERAL INTRODUCTION

1.1 Early development of the nervous system

Topographically the nervous system is comprised of two parts, the central and peripheral nervous system. The central nervous system (CNS) lies in the longitudinal axis of the body and comprises the brain and spinal cord, whilst the peripheral nervous system (PNS) consists of cranial and spinal nerves that arise from the brain and cord, and of the ganglionated trunks, plexuses and nerves which constitute the peripheral autonomic components of the nervous system. Vertebrate neurodevelopment is a multistage process that begins with neural induction in which the cells of the dorsal ectoderm are induced by the underlying precaudal mesoderm. A midline furrow arises along the anterior-posterior axis of the neural plate causing the lateral edges or the neural folds to elevate. The neural plate rounds up allowing the neural folds to meet in the dorsal midline, thus forming the intact neural tube. This is a process termed neurulation (Schoenwolf & Smith, 1990). The neural tube gives rise to the central nervous system, i.e. the brain and spinal cord. The fusion of the neural tube begins in the cervical region and proceeds in both cephalic and caudal directions. During the invagination of the neural plate, a group of cells migrate from the dorsal neural tube and emerge in a rostral to caudal sequence along the entire length of the axis of the neural groove (Weston, 1962). These cells are known as neural crest cells and form an intermediate zone between the neural tube and surface ectoderm. Neural crest cells give rise to most of the peripheral nervous system (PNS) including neurons of the dorsal root ganglia and cranial sensory nerves (proximal neurons of the trigeminal ganglia and the proximal ganglia of IX and X cranial nerves) post ganglionic neurons

of the sympathetic, autonomic and enteric nervous system and various supporting cells within the PNS such as Schwann cells (Weston, 1962).

Some cranial sensory neurons are derived from neurogenic placodes. These placodes are ectodermal thickenings that contribute neurons to the distal part of the trigeminal ganglia, geniculate, the vestibular cochlear and distal ganglia of the cranial nerves IX and X (Le Douarin, 1982). Whilst the majority of primary sensory neurons are located within spinal or cranial sensory ganglia, the neural crest derived proprioceptive neurons of the mesencephalic nucleus of the trigeminal nerve (TMN) are situated within the CNS.

1.2 Programmed cell death

During development, programmed cell death (apoptosis) is an active and widespread mechanism responsible for actively removing excess cells and cells that have served their purpose (Oppenheim et al., 1991; Williams, 1991). Cell death by apoptosis occurs when a cell activates an internally encoded suicide program following either extrinsic or intrinsic signals. Apoptotic cell death is characterized by plasma membrane blebbing, cytoplasmic condensation, pyknosis and degradation of nuclear DNA in to oligosomal lengths (Wyllie et al., 1980). Many extracellular signals that regulate apoptosis in different cell types have been identified, but less is known about the intracellular mechanisms that activate apoptosis within the cell. Early studies demonstrating that the inhibition of RNA or protein synthesis in various cell types was able to inhibit apoptosis suggested that apoptosis was dependent upon the activation of a program of gene expression within the dying cell (Cohen & Duke, 1984; Scott & Davies, 1990). Genetic analysis of the nematode *Caenorhabditis elegans* has led to the isolation of mutants defective for different steps in the apoptotic pathway. Mutation studies have identified ced-3 and ced-4 as being necessary for apoptosis as their inactivation results in increased cell survival (Ellis & Horvitz, 1986). In contrast, ced-9 appears to be necessary for cell survival, as a loss-of-function mutation of ced-9 results in extensive cell death via the ced-3 and ced-4 pathways (Hengartner et al., 1992). Ced-3 has been shown to encode an aspartate-specific cysteine protease (Yaun et al., 1993; Xue et al., 1996). Studies have shown the presence of several mammalian homologs of the nematode gene Ced-3 which have been termed 'caspases' (for cysteine aspase). The first mammalian homology identified was interleukin-1β (IL-1β) coverting enzyme (ICE), a cysteine protease responsible for the processing of pro-IL-1β to the active cytokine (Yuan et al., 1993). Overexpression of ICE or Ced-3 in Rat-1 fibroblasts induces apoptosis, suggesting that ICE is functionally and structurally related to Ced-3 (Miura et al., 1993). In contrast the cowpox virus CrmA gene product

and the baculovirus p35 protein both interfere with the protease function of ICE-related proteases and inhibit apoptosis (Tewari et al., 1995; Xue et al., 1995; Miura et al., 1995; Bump et al., 1995) suggesting that ICE-related proteases are involved in apoptotic pathways. Eight mammalian homologs of Ced-3 and ICE have been characterized: ICH-1 (Kumar et al., 1994; Wang et al., 1994b), CPP-32 (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), ICH-2 (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), ICErel-111 (Munday et al., 1995) Mch-2 (Fernandes-Alnemri et al., 1995a), Mch-3 (Duan et al., 1996; Fernandes-Alnemri et al., 1995b; Lipkė et al., 1996), FLICE (Muzio et al., 1996) and ICE-LAP6 (Duan et al., 1996). Ectopic expression of these ICE/Ced-3 homologs in a variety of cells causes apoptosis. All caspases cleave their substrates after specific aspartic acids and are themselves activated by cleavage at specific aspartic acids. Caspases mediate apoptosis by cleaving selected intracellular proteins, including proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol. Some of the cleaved proteins activate other destructive processes within the cell and thereby enhance apoptosis (Chinnaiyan & Dixit, 1996). Ced-9 has since been identified as a homolog of the mammalian proto-oncogene Bcl-2 (Hengartner & Horvitz, 1994). Overexpression of Bcl-2 is able prevent the ectopic cell death that occurs in ced-9 loss-of-functions mutants (Hengartner & Horvitz 1994), demonstrating that the molecular mechanism of apoptosis is conserved through out evolution.

The Bcl-2 gene was first isolated from the breakpoint of the t(14;18) chromosmal translocation found in many human B cell lymphomas (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary & Sklar, 1985). This translocation places the Bcl-2 gene under the influence of a powerful enhancer element in the immunoglobulin (Ig) heavy chain locus, resulting in a marked overexpression of Bcl-2 in B cells and the development of neoplasia due to the suppression of cell death (Seto et al., 1988). The Bcl-2 protein is an intracellular integral membrane protein with a molecular mass of 26

KD (Chen-Levy et al., 1990). Bcl-2 has been shown to play an important role in regulating the survival of B and T lymphocytes within the immune system (reviewed in Linette & Korsmeyer, 1994). During development, Bcl-2 expression in B and T cell lines is downregulated during periods of increased proliferation, but upregulated during positive selection of immature B and T cells. Mice containing a targeted null mutation in the Bcl-2 gene show a marked reduction of B and T cells as a result of increased apoptosis (Strasser et al. 1991; Nakayama et al., 1993, 1994; Veis et al., 1993), whereas mice carrying a transgene resulting in overexpression of Bcl-2 within the immune system show extended survival of B and T cells (McDonnell et al., 1989, 1990). However, overexpression of Bcl-2 cannot prevent the death of haematopoeitic cell lines deprived of interleukin –2 (IL-2) or IL-6, although it can rescue cells deprived of IL-3, IL-4 or granulocyte macrophage colony stimulating factor (Nunez et al., 1990).

Bcl-2 has also been shown to be capable of promoting the survival of neurons. For example, over expression of Bcl-2 reduces the death of cultured sympathetic and sensory neurons which have been deprived of growth factors (Garcia et al., 1992; Allsopp et al., 1993). DRG neurons from mice that constitutively overexpress Bcl-2 show extended survival in vitro without trophic support, as compared to wild-type neurons (Farlie et al., 1995). Overexpression of Bcl-2 also prevents motor neuron death induced by facial nerve axotomy (Dubois-Dauphin et al., 1994) and sciatic nerve axotomy (Farlie et al., 1995). Conversely, reduction or elimination of endogenous Bcl-2 reduces neuronal survival both in vitro and in vivo (Allsopp et al., 1995; Michaelidis et al., 1996; Pinon et al., 1997). Bcl-2 does not have a universal role in promoting neuronal cell survival, since overexpression of Bcl-2 cannot rescue parasympathetic neurons following CNTF deprivation (Allsopp et al., 1993).

Bcl-2 is the founder member of a family of cytoplasmic proteins that modulate the response of many cell types to the diverse extracellular signals that affect their

survival (Davies, 1995). While some members of the family are anti-apoptotic, other family members appear to promote apoptosis. Among the death inhibitors are Bcl-2 (Hockenbery et al., 1990), Bcl-xL (Boise et al., 1993), Bcl-w (Gibson et al 1996), McI-1 (Reynolds et al., 1994), A-1 (Lin et al., 1993), adenovirus EIB 19K (Han et al., 1996), Epstein-Barr virus (EBV) BHRF1 (Henderson et al., 1993) and CED-9 (Hengartner & Horvitz, 1994). Pro-apoptotic members include Bax (Oltvai et al., 1993), Bik (Boyd et al., 1995), Bid (Wang et al., 1996), Bim (O'Connor et al., 1998), Bok/Mtd (Hsu et al., 1997; Inohara et al., 1998) Hrk (Inohara et al., 1997), Bak (Chittenden et al., 1995), Bcl-xs (Minn et al., 1996) and Bad (Yang et al., 1995). The role of Bax in mediating neuronal survival has been well characterized. The overexpression of Bax accelerates neuronal death following neurotrophin withdrawal (Vekrellis et al., 1997; Martinou et al., 1998). In contrast, the reduction or elimination of endogenous Bax promotes the survival of neurons without neurotrophic factors in culture and prevents their death in vivo (Deckwerth et al., 1996; Gillardon et al., 1996; Miller et al., 1997; White et al., 1998).

Bcl-2 related proteins share homology in four regions designated the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4, with the greatest similarity in the proteins encoded by the genes of the Bcl-2 family being observed in BH1 and BH2. Mutational analysis of Bcl-2 suggests that both BH1 and BH2 domains are required for the anti-apoptotic function of members of the Bcl-2 family (Yin et al., 1994). However, recent studies have shown that while all four homology regions are present in some family members, such as Bcl-2 and Bcl-x_L, many other proteins lack one or more of the domains. For example, certain pro-apoptotic proteins, such as Bad and Bid, contain only the BH3 domain, suggesting that the BH3 domain is a potent death domain (Kelekar & Thompson, 1998). In addition, members of the Bcl-2 family contain a less well conserved 19 amino acid hydrophobic C-terminus domain which is thought to be important for the attachment of the proteins to the cytosolic side of cell

membranes (Nguyen et al., 1993; Tanaka et al., 1993). Immunoprecipitation and yeast two hybrid studies have demonstrated that Bcl-2 family members are capable of forming either homodimers or heterodimers (Sato et al., 1994). For example, the pro-apoptotic protein Bax associates in vivo as a heterodimer with the anti-apoptotic protein Bcl-2 as well as forming homodimers (Oltvai et al., 1993;). It has been proposed that it is the ratio of pro-apoptotic to anti-apoptotic proteins (rheostat model) that determines whether a cell survives or undergoes apoptosis following an apoptotic signal (Korsmeyer et al., 1993). Site directed mutagensis has shown that the mutation of Gly145 in the BH1 domain or Trp 188 in the BH2 domain of Bcl-2 prevents Bcl-2 from forming heterodimers with pro-apoptotic proteins. These mutations inactivate the anti-apoptotic action of Bcl-2, but do not prevent the formation of Bcl-2 homodimers (Yin et al., 1994). These findings suggest that Bax/Bcl-2 heterodimers prevent cell death by reducing the number of Bax homodimers.

The mechanism by which Bcl-2 acts to inhibit apoptosis is unclear and controversial. Numerous mechanisms have been proposed based on the intracellular localization of Bcl-2 to the outer mitochondrial membrane, endoplasmic reticulum (ER) membrane and nuclear envelope (Chen-Levy et al., 1989; Hockenbery et al., 1990; Jacobson et al., 1993). The localization of Bcl-2 to the mitochondrial membrane suggests the possibility that it might protect against apoptosis by altering mitochondrial function. However, mutant cell lines which lack mitochondrial DNA continue to undergo apoptosis following serum deprivation, and are protected from apoptosis by overexpression of Bcl-2 (Jacobson et al., 1993). The presence of Bcl-2 within the membranes of the endoplasmic reticulum (ER) raises the possibility that Bcl-2 might regulate intracellular calcium levels, since release of calcium from ER stores accompanies apoptosis in certain cells. In line with this, overexpression of Bcl-2 in lymphoma cells reduces the efflux of calcium from the ER and prevents apoptosis (Lam et al., 1994). However, studies in a neuronal cell line suggests that a rise in the

intracellular concentration of free calcium is not a prerequisite for apoptosis (Zhong et al., 1993b). The presence of Bcl-2 at major sites of oxygen free radical generation, together with the demonstration that reactive oxygen species might be involved in causing apoptosis in different cell types (Buttke & Sandstrom, 1994), has raised the possibility that Bcl-2 might prevent apoptosis by acting either as an antioxidant or by inhibiting production of free radicals (Kane et al., 1993; Kinouchi et al., 1991). Another theory is that Bcl-2 related proteins may play a role in intracellular signal transduction, since Bcl-2 is phosphorylated on serine residues in response to a variety of stimuli (May et al., 1994; Halder et al., 1995; Chen & Faller, 1996). Phosphorylation of Bcl-2 is associated with a loss of its ability to promote survival. Bcl-2 has been shown to associate with p72Raf1, a serine/threonine specific protein kinase (Wang et al., 1994), and p23-R-Ras a GTPase member of the Ras family (Fernandez & Sarabia, 1995) which promotes apoptosis following growth factor removal (Wang et al., 1995). Another potential mechanism by which Bcl-2 may function has been suggested by the analysis of the 3D structure of Bcl-x_L. Bcl-x_L comprises two central hydrophobic α helices surrounded by five amphipathic helices, as well as a 60 residue flexible loop (Muchmore et al., 1996). This tertiary structure shows a high degree of homology with the pore-forming domains of certain bacterial toxins, such as colicins A and E1 and diphtheria toxin, that act as ion channels (Muchmore et al., 1996). It is thought that anti-apoptotic protiens such as Bcl-2 and Bcl-x_L may form pores involved in homeostasis which may protect the cell against electrochemical changes which occur under stressful conditions, whilst pro-apoptotic family members might interfere with the assembly or the function of such pores, thus eliminating the ability of anti-apoptotic family members to protect cells against cell death (Minn et al., 1997; Schlesinger et al., 1997; Schendel et al., 1997).

There is growing evidence that some members of the Bcl-2 family control the release of cytochrome c from the mitochondria which promotes a cascade of caspase

activation by interacting with the adaptor protein apf-1 which in turn activates pro-caspase-9 (Li et al., 1997b; Qin et al., 1999). Pro-apoptotic members of the family like Bax and Bak increase mitochondrial permeability allowing cytochrome c to pass into the cytosol, whereas anti-apoptotic members like Bcl-2 and Bcl-xL prevent cytochrome c release (Kharbanda et al., 1997; Kluck et al., 1997; Yang et al., 1997; Shimizu et al., 1999). Inhibiting the release of cyctochrome c from mitochondria cannot entirely account for the means by which pro-apoptotic members of the Bcl-2 family prevent apoptosis because overexpression of these proteins can prevent apoptosis in cells in which cytochrome c is present in the cytosol (Li et al., 1997a; Rosse et al., 1998). There is evidence that Bcl-2 can regulate the activity of membrane associated pro-caspase-3 independently of cytochrome c (Krebbs et al., 1999) and Bcl-xL can bind directly to Apf-1 preventing it from activating pro-caspase-9 (Hu et al., 1998; Pan et al., 1998).

Examination of the expression pattern of the Bcl-2 protein has revealed that it is widely expressed in the developing nervous system during embryonic development. In the PNS, neurons and supporting cells of sympathetic and sensory ganglia all exhibit high levels of Bcl-2 expression during development which is retained throughout life (Merry et al., 1994). In the developing CNS, Bcl-2 expression is observed in numerous structures including proliferating neuroepithelial cells of the ventricular zones as well as the postmitotic cells of the cortical plate, cerebellum, hippocampus and spinal cord (Abe-Dohmae et al., 1993; Merry et al., 1994). Unlike the PNS, Bcl-2 expression in the CNS decreases with age, with postnatal expression only being observed in the granule cells of the cerebellum and the dentate gyrus of the hippocampus (Merry et al., 1994). Bcl-2 expression has also been shown to be widespread in various non-neuronal cell types and tissues characterized by high cell turnover, such as the spleen and thymus (Negrini et al., 1987; Hockenbery et al., 1991), duct cells in exocrine

glands, basal keratinocytes, cells located at the bottom of colon crypts and neurons (Abe-Dohmae et al., 1993; Lu et al., 1993)

Mice containing a homozygous null mutation of the Bcl-2 gene are viable at birth but die between two and ten weeks after birth (Nakayama et al., 1993; Veis et al., 1993; Kamada et al., 1995). Bcl-2 deficient mice develop polycystic kidney disease and have elevated levels of urea and creatinine in their blood compared to their wild-type and heterozygous litter mates. Bcl-2 deficient mice also exhibit a massive involution of the spleen and thymus as well as developing hypo-pigmented hair (Nakayama et al., 1993; Veis et al., 1993; Kamada et al., 1995). There is also a reduction in the number of oocytes and primordial follicles in postnatal females, suggesting a role for Bcl-2 in preventing follicular atresia (Ratts et al., 1995). Surprisingly, certain tissues, including intestine, skin and the nervous system, show no gross abnormalities in Bcl-2 deficient mice despite the fact that these tissue show high levels of Bcl-2 expression in wildtype mice.

1.3 The Neurotrophic Theory

Neurons are initially generated in excess during neurodevelopment. Shortly after neurons begin to innervate their target tissue, large numbers die by apoptosis (Oppenheim, 1991). The neurotrophic theory states that neuronal target fields regulate the number of neurons surviving during the phase of target field innervation by synthesizing limited quantities of a neurotrophic factor that the innervating neurons require for survival (Davies, 1988a,b; Purves, 1988; Barde 1989). The most important evidence to support the neurotrophic theory has come from experiments that have altered the availability of NGF to neurons both in vitro and in vivo. Such experiments have demonstrated that sympathetic and certain sensory neurons require NGF to survive during the period of target field innervation (Levi-Montalcini, 1987; Crowley et al., 1994).

Further evidence for the role of neurotrophic factors in regulating neuronal survival has come from the demonstration that NGF is synthesized within the target field of sensory and sympathetic neurons during development. NGF synthesis commences at the onset of target field innervation (Davies et al., 1987a; Korsching & Thoenen, 1988) and the amount of NGF synthesized is in proportion to the final innervation density of the target field (Harper & Davies, 1990). Specific neuronal cell surface receptors mediate rapid uptake of NGF within the target field. NGF is transported by retrograde axonal transport to the cell bodies of the innervating neurons where NGF exerts its survival promoting effects (Korsching & Thoenen, 1983a; Palmatier et al., 1984; Davies et al., 1987a). The generality of the neurotrophic hypothesis was established following the cloning and purification of brain derived neurotrophic factor (BDNF), a protein with extensive sequence homology to NGF (Barde et al., 1982; Leibroch et al., 1989). BDNF has been shown to promote the survival of certain populations of sensory neurons in vitro (Lindsay et al., 1985; Davies et al 1986a,b) and to prevent naturally occurring cell death in vivo following BDNF administration to chicken embryos during the period of naturally occurring cell death (Hofer & Barde, 1988).

More recently NGF has been shown to be the founder member of a family of six structurally related homologous proteins termed "the neurotrophins" which also include NT-3 (Ernfors et al., 1990a, Hohn et al., 1990; Jones & Reichardt 1990; Maisonpierre et al., 1990b Rosenthal et al., 1990), NT-4/5 (Berkemeier et al., 1991; Hallbook et al 1991; Ip et al 1992), NT-6 (Gotz et al., 1994) and NT-7 (Nilsson et al., 1998; Ko et al., 1998). These proteins will be discussed in turn.

Nerve Growth Factor

The presence of large quantities of NGF within the adult male mouse submandibular gland facilitated its purification and characterization of its primary

structure (Cohen 1960, Angeletti & Bradshaw 1971, Angeletti et al 1973). Other sources of NGF include exocrine tissues and their secretions, such as snake venom (Cohen, 1959; Bailey et al., 1975; Oda et al., 1989) and the prostate glands of guinea pig, rabbit and bull (Harper et al., 1979, Harper & Thoenen, 1980). Analysis of the cDNA sequence of murine NGF has shown that the mature NGF protein is generated following proteolytic cleavage of a 305 amino acid precursor protein termed pre-proNGF (Scott et al., 1983; Ullrich et al., 1983). The NGF gene has been cloned from several species, with nucleotide sequence analysis showing that the mature NGF protein has been highly conserved during evolution (Scott et al., 1983; Ullrich et al., 1983; Meier et al., 1986; Ebendal et al., 1986; Selby et al., 1987; Whittemore et al., 1988; Schwarz et al., 1989). NGF derived from the mouse submandibular gland is found as an acidic protein complex termed 7sNGF with a molecular weight of 130 KD. This is composed of 3 subunits termed α , β and γ (Varon et al 1968). Only the β subunit has been shown to possess neurotrophic activity and is a basic protein composed of two identical 118 amino acid chains (Greene et al., 1969, Angeletti et al., 1971; Smith et al., 1969). The γ subunit, an arginine esteropeptidase, is thought to be involved in the processing of the β NGF. The α subunit shows no enzymic activity and is believed to protect β NGF from enzymatic degeneration, as well as regulating the activity of both the β and γ subunits. The mouse gene encoding βNGF is 45Kb in length and consists of 5 exons separated by 4 introns. The mature protein is encoded from a single 3' exon within the NGF gene (Ullrich et al., 1983, Edwards et al., 1986). The remaining exons are smaller and undergo alternative splicing to produce at least 4 transcripts which encode precursor proteins that differ in their amino termini (Selby et al., 1987).

In vivo and *in vitro* studies have demonstrated that NGF can exert a variety of trophic effects on neurons including regulating neuronal survival during development, maintaining the phenotype of mature neurons and enhancing the regeneration of injured

neurons of the PNS (Reviewed by Thoenen & Barde 1980, Levi-Montalcini, 1987, Lindsay, 1988, Barde, 1989). In particular, NGF has been shown to support the survival of sympathetic neurons and a subpopulation of neural crest-derived sensory neurons during development (Cohen & Levi-Montalcini, 1956; Cohen, 1960; Thoenen & Barde, 1980; Davies & Lindsay, 1985; Levi-Montalcini, 1987; Davies, 1994c). Within the CNS, NGF exerts trophic effects on cholinergic neurons of the basal forebrain (Williams et al., 1986; Hartikka & Hefti, 1988; Knusel et al., 1990). *In vitro* and *in vivo* studies have shown that NGF promotes an increase in axonal branching and terminal and dentritic arborization in neonatal and adult sympathetic and sensory neurons, whilst NGF deprivation results in a reduction in both terminal and dentritic arborizations (Bjerre et al., 1975a,b; Snider, 1988, Ruit et al., 1990; Ruit & Snider, 1991; Yasuda et al 1990; Scott & Davies, 1993).

Northern blot analysis and in situ hybridization have shown that NGF mRNA is expressed at high levels in the target tissues innervated by NGF responsive sympathetic and sensory neurons (Korsching & Thoenen, 1983; Korsching et al., 1985; Heumann et al., 1984; Shelton & Reichardt 1984). The identity of the cells that synthesize NGF has been determined in a detailed developmental study of mouse whisker pad, the target field for developing trigeminal sensory neurons (Davies et al., 1987a). Enzymatic dissociation of the developing whisker pad into its principle components, the mesenchyme (presumptive dermis) and epithelium (presumptive epidermis) and subsequent analysis of NGF expression revealed that the highest levels of NGF mRNA are found in the epithelium (Davies et al., 1987a). Subsequent analysis has shown that the levels of NGF mRNA expressed within the peripheral target tissues of sensory neurons during the period of neuronal death is proportional to their final innervation densities (Harper & Davies, 1990). The onset of NGF synthesis within the peripheral target fields of sensory and sympathetic neurons coincides with the onset of target field innervation which suggests that NGF regulates the number of neurons

innervating peripheral targets after the target tissue has been contacted (Korsching & Thoenen, 1983; Harper & Davies, 1990).

Analysis of mice that are homozygous for a null mutation of the NGF gene has helped to determine the role that NGF plays in the development of the nervous system in vivo. NGF -/- mice are viable at birth, but do not gain weight in comparison with their wildtype littermates and die within the first month of life (Crowley et al., 1994). They also fail to respond to noxious mechanical stimuli. Histological examination has shown that NGF deprivation results in severe neuronal cell loss within sensory trigeminal and dorsal root sensory ganglia by birth and a complete absence of sympathetic ganglia by postnatal day 10 (Crowley et al., 1994). Histological comparison of DRG ganglia from wildtype and NGF deficient mice has shown that null mutant mice exhibit a complete absence of small diameter DRG neurons as well as a reduction in the number of neurons expressing CGRP or substance P immunoreactivity. This observation supports the view that NGF is required for the development of small peptidergic DRG neurons which are thought to mediate pain (nociceptive and thermal receptive functions) (Crowley et al., 1994). These findings are consistent with earlier antibody deprivation studies which showed that anti-NGF antibodies administrated during the phase of target field innervation eliminated sensory and sympathetic neurons, and these neurons could be rescued by exogenous NGF (Levi-montalcini & Angeletti, 1968; Johnson et al., 1980; Hamburger & Yip, 1984; Carrol et al., 1992; Ruit et al., 1992). In contrast, transgenic mice overexpressing NGF within the epidermis exhibit hypertrophy of peripheral sensory and sympathetic neurons and an increase in the expression of CGRP. In addition, both trigeminal and superior cervical ganglia contain more neurons in NGF overexpressing mice, indicating a reduction of programmed or naturally occurring cell death (Albers et al., 1994). Transgenic mice expressing high levels of NGF mRNA also exhibit profound hyperalgesia to noxious mechanical stimulation (Albers et al., 1994). In contrast to

previous observations (Hartikka & Hefti, 1988a,b), NGF null mutant mice show no loss of basal forebrain cholinergic neurons which maintain their phenotype throughout the limited life span of the NGF null mice. Basal cholinergic neurons of NGF knockout mice do, however, display lighter staining for the transmitter biosynthesis enzyme cholinecetyltransferase (ChAT) when compared to wildtype mice (Crowley et al., 1994). These results are consistent with observations which show that NGF increases (Hartikka & Hefti, 1988a) and anti-NGF decreases (Vantini et al., 1989) ChAT expression in basal forebrain cholinergic neurons.

Brain Derived Neurotrophic Factor

BDNF is the second member of the NGF family of neurotrophins to be identified and was initially purified from pig brain (Barde et al., 1982). BDNF is a basic protein (pI $\geq$ 10.1) with a molecular weight of 12.3 KD (Barde et al., 1982). Sequence analysis of the BDNF cDNA has shown that mature BDNF is synthesized as a precursor protein containing a single peptide, an amino-terminal precursor region and a highly conserved carboxyl terminal domain. Proteolytic processing of the precursor protein releases a 119 amino acid mature form of BDNF (Leibrock et al., 1989). Like NGF, biologically active BDNF exists as a tightly associated homodimer (Radziejewski et al., 1992). The rat BDNF gene is contained within 4.0Kb of genomic DNA and consists of four 5' exons, linked to separate upstream promoters, and one 3'exon which encodes the mature BDNF protein. Alternative promoter usage and differential splicing results in eight different BDNF mRNAs with four different 5' untranslated exons. BDNF mRNAs containing exons I, II, and III are expressed predominantly in the brain, whilst transcripts containing exon IV predominate in the

lung and heart (Timmusk et al., 1993a; Metsis et al., 1993). The porcine, murine and human genes encoding BDNF have also been cloned and the primary structures determined (Leibrock et al., 1989; Hofer et al., 1990; Maisonpierre et al., 1991). Comparison of the primary structures of BDNF and NGF has revealed a 55% homology with 51 identical amino acids. BDNF also shows a high degree of homology between different species. Included in the conserved residues between BDNF and NGF sequences are all 6 cysteine residues that form disulphide bridges, suggesting that the 3-dimensional structure of BDNF is similar to NGF (Leibrock et al., 1989).

BDNF has been shown to support the survival of several populations of central and peripheral neurons. In the PNS, BDNF has been shown to promote the survival of certain sensory neurons (Lindsey et al., 1985; Davies et al., 1986b; Davies et al., 1987b; Hofer & Barde 1988). In the CNS, BDNF has been shown to support the survival of embryonic mesencephalic dopaminergic neurons in the substantia nigra (Hyman et al., 1991; Knusel et al., 1991), basal forebrain cholinergic neurons (Alderson et al., 1990), retinal ganglion cells (Johnson et al., 1986) embryonic motor neurons (Oppenheim et al., 1992; Yan et al., 1992; Sendtner et al., 1992; Koliatsos et al., 1993) and pyramidal neurons of the visual cortex (McAllister et al., 1995, 1996,1997).

Northern blot analysis of BDNF mRNA expression in adult mice and rats has shown that the CNS is the predominant site of BDNF mRNA expression (Liebrock et al., 1989; Hofer et al., 1990; Maisonpierre et al., 1990a, Yan et al., 1997). The highest levels of BDNF mRNA are found within the cerebellum, cerebral cortex and the hippocampus, where it is confined to a subset of pyramidal and granular neurons. Detectable levels of BDNF mRNA are also observed in the central target fields of BDNF responsive sensory neurons, namely the spinal cord and hindbrain (Ernfors et al., 1990a; Ernfors et al., 1990b; Hofer et al., 1990; Philips et al 1990). In the peripheral nervous system, BDNF mRNA expression has been detected in developing

sensory neurons within dorsal root, trigeminal and jugular ganglia as well as in sympathetic ganglia (Ernfors et al., 1990; Schecterson & Bothwell, 1992; Wright et al., 1992; Robinson et al., 1996b). The observation that BDNF expression is largely restricted to developing NGF dependent neurons during the period of naturally occurring neuronal death rules out a BDNF autocrine loop supporting the survival of embryonic neurons and suggests that BDNF functions in a paracrine manner (Robinson et al., 1996a). A BDNF autocrine loop does promote the early maturation of newly differentiated sensory neurons (Wright et al., 1992) and supports survival of a subset of adult DRG neurons (Acheson, et al., 1995). Low levels of BDNF mRNA have also been detected in heart, lung and muscle (Maisonpierre et al., 1990a; Schecterson & Bothwell, 1992). Increased expression of BDNF mRNA containing exons I, II, and III has been demonstrated in hippocampal neurons following addition of the glutamate analog, kainic acid (KA) and the γ-aminobutyric acid subtype A ($GABA_A$) receptor antagonist bicuculline, as well as stimulation of the septohippocampal pathway. This suggests that the levels of BDNF mRNA expressed by hippocampal neurons is controlled by the balance between the activity of GABAergic and glutamatergic systems (Zafra et al., 1991, Zafra et al., 1990).

Mice that are homozygous for a null mutation in the BDNF gene are viable at birth. Most mutants die within 2 days of birth, although some may live for 2-4 weeks. BDNF null mutants exhibit severe deficiencies in coordination and balance, which is believed to be associated to the increase neuronal loss in several sensory ganglia including the vestibular ganglia (Ernfors et al., 1994a; Jones et al., 1994). Histological analysis of BDNF null mice revealed significant neuronal loss in trigeminal, geniculate, vestibular and nodose ganglia (Ernfors et al., 1994a; Jones et al., 1994). Contrary to previous studies showing that BDNF can promote the survival of motor, hippocampal, dopaminergic and cortical neurons, no obvious defects were seen in these populations in the absence of BDNF. A possible explanation for this is that the function of BDNF

is compensated for by other neurotrophins in the mutant mice. For example, BDNF, NT-3 and NT-4 have all been shown to promote motor neuron survival in vitro (Henderson et al., 1993).

Although the gross morphology of the CNS of BDNF mice showed no abnormalities, histological examination of BDNF mutants showed reduced expression of neuropeptide Y (NPY) and the calcium binding proteins paravalbumin and calbindin (Ernfors et al., 1994b; Jones et al., 1994). Due to the fact that BDNF mutants exhibit early postnatal lethality, they may not survive long enough for a wider phenotype to be observed. These observations suggest that BDNF is an essential factor for the survival of certain sensory neuronal populations, but is not essential for the early development of the central nervous system (Ernfors et al., 1994a; Jones et al ., 1994).

Neurotrophin-3

NT-3 was first isolated by the polymerase chain reaction (PCR) using degenerate primers whose sequences were based on the nucleotide sequences of conserved domains of NGF and BDNF. NT-3 has since been isolated and cloned in mouse, rat, and humans (Hohn et al., 1990; Maisonpierre et al., 1990a; Jones & Reichardt 1990). Structural analysis of NT-3 has revealed that it is initially synthesized as a precursor protein of 258 amino acids. Mature NT-3 is predicted to consist of 119 amino acids and be a basic protein (pI $\geq$ 9.3) with a molecular weight of 13.6 KD. Comparison of the mature form of NT-3 with other members of the neurotrophin family has revealed a high degree of sequence homology (57%-48%) with 54 amino acids being conserved, including all six disulphide linked cysteine residues (Hohn et al., 1990).

The NT-3 gene consists of two small upstream exons, termed IA, and IB, and a large downstream exon termed exon II, which encodes the mature NT-3 protein (Leingartner & Lindholm, 1994). Two classes of NT-3 transcripts have been

identified, termed transcripts A and B, which are generated by alternative splicing of exons IA or exon IB to the common exon II. The NT-3 gene also contains within both upstream exons several transcription start sites. Exon II, contains three different polyadenylation sites, which give rise to multiple NT-3 mRNA variants which differ slightly in length (Leingartner & Lindholm, 1994).

Northern blot analysis has revealed that NT-3 is expressed in various tissues including heart, kidney, spleen, and lung. NT-3 is also expressed in several regions of the adult CNS including the cerebellum, medulla oblongata and hippocampus (Hohn et al., 1990; Jones & Reichardt et al., 1990; Maisonpierre et al., 1990a,b; Rosenthal et al., 1990). The broad spectrum of peripheral tissues containing NT-3 mRNA suggests that in the PNS, NT-3 could serve as a target-derived factor for sympathetic and sensory neurons. In situ hybridization has been used to further determine the spatio-temporal expression pattern of mRNA. NT-3 transcripts are present in the iris, cochlea and vestibular organs of embryonic and early postnatal rats, as well as in the epithelium of the whisker follicles, the target of trigeminal neurons (Ernfors et al., 1992; Pirvola et al., 1992; Hallbook et al., 1993). Northern blot analysis has demonstrated that NT-3 mRNA is expressed in the peripheral trigeminal target field before the arrival of the earliest axons, with the expression of NT-3 mRNA peaking during the early stages of target field innervation and declining following the loss of neuronal responsiveness to BDNF and NT-3 (Buchman & Davies, 1993). Within the CNS, NT-3 expression appears to be more restricted than other members of the neurotrophin family, with NT-3 mRNA being detected in the dentate gyrus of the hippocampus, cerebellum (Ernfors et al., 1990b; Hohn et al., 1990), nucleus basalis and the visual cortex (Jones et al., 1990).

In the PNS, NT-3 has been shown to promote the differentiation of sensory neurons from their progenitors in vitro, as well as enhancing early maturational changes in dorsal root ganglion neurons before they become dependent on

neurotrophins for survival (Wright et al., 1992; Buchmann & Davies, 1993; Elshamy et al., 1996). NT-3 also supports the survival of spinal sensory and sympathethic neurons (Maisonpierre et al., 1990; Rosenthal et al., 1990 Wyatt et al., 1997). However, among DRG's, NT-3 most effectively promotes the survival of lumbar and cervical neurons which contain many proprioceptive neurons (Hory-Lee et al., 1993). In vivo and in vitro studies have also shown that NT-3 is required for the survival of developing muscle sensory neurons (Hohn et al., 1990; Ericksson et al., 1994; Oakley et al., 1995). Several additional populations of sensory neurons have been shown to respond to NT-3, these include neurons from the trigeminal (Buchman & Davies, 1993; Wilkinson et al., 1996) and nodose ganglia (Gaese et al., 1994; Ockel et al., 1996) and neurons of the spiral ganglia (Gao et al., 1995; Ernfors et al., 1995). Recently, NT-3 has been demonstrated to be required for survival of a subset of sympathetic neurons that also require NGF (Francis et al., 1999). Functional analysis of NT-3 within the CNS, has shown that NT-3 supports the survival of certain neuronal populations and certain glia. For example, NT-3 stimulates expression of c-fos in hippocampal neurons and promotes their survival (Collazo et al., 1992; Ohsawa et al., 1993). NT-3 also promotes the survival of oligodendrocyte precursors in the embryonic rat hippocampus (Bertollini et al., 1997).

NT-3 has also been shown to promote neurite out growth within different neuronal populations. NT-3 stimulates dendritic growth of pyramidal neurons within the developing visual cortex (McAllister et al., 1995; 1997). In addition, NT-3 promotes neurite outgrowth from embryonic sensory neurons and supports the development of distinct axon morphologies (Dijkhuizen et al., 1997; Lentz et al., 1999). NT-3 enhances the sprouting from the corticospinal tract during development and after adult spinal cord lesions (Schnell et al., 1994). Mice containing a homozygous null mutation for the NT-3 gene are viable at birth but exhibit a severe defect in limb movement suggesting deficits in proprioception. Most NT-3 null mice

die shortly after birth (Ernfors et al., 1994; Farinas et al., 1994; Tessarollo et al., 1994). Significantly, it has been shown that the main components of the proprioceptive system, (muscle spindles, Golgi tendons and large diameter myelinated dorsal root sensory neurons) are completely missing in NT-3 deficient mice (Ernfors et al., 1994b; Carr & Nagy, 1993). Interestingly, muscle spindles and Golgi tendon organs are reduced by 50% in NT-3 heterozygous mice, suggesting that the concentration of NT-3 is limiting during development (Ernfors et al., 1994b). NT-3 deficient mice also exhibit severe neuronal losses in nodose, trigeminal, superior cervical and spiral ganglia (Ernfors et al., 1994b; Tessarollo et al., 1994; Bovolenta et al., 1996; Farinas et al., 1996; Fritzsch et al., 1997; Wyatt et al., 1997). Neuronal cell loss has been observed in the dorsal root ganglion before the onset of the period of programmed cell death, suggesting that NT-3 prevents the death of proliferating sensory neuron precursor cells (EIShamy & Ernfors, 1996). Selective expression of NT-3 in skeletal muscle of mice lacking endogenous NT-3 rescues proprioceptive neurons (Wright et al., 1997). Cutaneous overexpression of NT-3 increases the number of sensory and sympathetic neurons in peripheral ganglia in addition to enlarging touch dome mechanoreceptor units and increasing hair follicle innervation (Albers et al., 1996).

Neurotrophin 4/5

NT-4/5 is the fourth member of the neurotrophin family to be identified. NT-4/5 was discovered and isolated from *Xenopus learis* following a phylogenetic analysis of the DNA sequences of members of the neurotrophin family (Hallbook et al., 1991). NT-4 is a 13.9 kD protein derived from a 236 amino acid precursor which is processed into the 123 basic amino acid mature NT-4 protein. NT-4 exhibits 50-60% amino acid homolgy with members of the neurotrophin family, including all six disulphide linked cysteine residues (Hallbook et al., 1991). Homologues of human and Xenopus NT-4 have subsequently been isolated from rat and termed mammalian NT-4 (Ip et al.,

1992a) or NT-5 (Berkemeier et al., 1991). It appears as if both proteins are identical and the mammalian equivalent of Xenopus NT-4 is often referred to as NT-4/5. The human NT-4 gene has been localized to chromosome 19, band q13.3 (Berkemeier et al., 1992). Northern blot analysis of Xenopus tissue only detected NT-4 mRNA in the ovary (Hallbook et al., 1991). However RNAase protection assay and Northern blot analysis have revealed that NT-4/5 mRNA is widely expressed both in the central nervous system and in peripheral organs in developing and adult rat tissue (Berkemeier et al., 1991; Ip et al., 1992; Timmusk et al., 1993). In the CNS, NT-4/5 mRNA is expressed in several regions including the cerebral cortex, brain stem, hippocampus, hypothalamus, thalamus and cerebellum. In the periphery, NT-4/5 mRNA is expressed in the lung, kidney, thymus, muscle, heart and stomach (Berkemeier et al., 1991; Timmuk et al., 1993b). During development, the peak of NT-4/5 mRNA expression in peripheral tissue coincides with the peak of naturally occurring neuronal cell death in peripheral ganglia, suggesting that NT-4/5 may function as a target-derived trophic factor in vivo.

Functional analysis of the neurotrophic activity of NT-4/5 in the PNS has shown that NT-4/5 transiently supports mouse NGF-dependent trigeminal and jugular neurons at early stages of target field innervation, as well as mouse BDNF-dependent enteric neurons of the nodose ganglia during the phase of naturally occurring cell death (Davies et al., 1993; Ibanez et al., 1993). Other peripheral neurons supported by NT-4/5 include dorsal root and paravertebral sympathetic neurons (Hallbook et al., 1991) and postnatal spiral ganglion and vestibular neurons (Zheng et al., 1995a; Zheng et al., 1995b). Mammalian NT-4/5 does not support the survival of chicken BDNF-dependent dorsomedial (DMTG), ventrolateral trigeminal (VLTG) neurons or trigeminal mesencephalic (TMN) sensory neurons (Davies et al., 1993). A chick NT-4/5 homologue has not yet been isolated. In the CNS, NT-4/5 supports the survival of developing and adult motor neurons (Henderson et al., 1993; Koliatsos et al., 1994;

Freidman et al., 1995; Funakoshi et al., 1995) and corticospinal neurons of neonatal rats (Junger & Varon, 1997). NT-4/5 also promotes the development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla (Schober et al., 1998). Furthermore NT-4/5 has also been shown to support the survival and, or differentiation of numerous sub-populations of neurons within the CNS including those from the substantia-nigra, striatum and cerebellum (Gao et al., 1995; Ventimilglia et al., 1995; Studer et al., 1995). NT-4/5 also supports adult rat retinal ganglion cell survival (Cohen et al., 1995).

Mice homozygous for a null mutation of the NT-4/5 gene are viable at birth, but display a loss of sensory neurons in the nodose-petrosal and geniculate ganglia. In contrast, there is no loss of neurons from the facial motor nucleus, ciliary ganglion or the substantia nigra (Conover et al., 1995; Liu et al., 1995; Erickson et al., 1996).

Neurotrophin-6 (NT-6)

NT-6 was cloned from a genomic library of the platyfish *Xiphophorus maculatus*, during attempts to clone the fish NGF gene (Gotz et al., 1994). NT-6 is a 16 KD protein derived from a 286 amino acid precursor protein, with a molecular weight of 31.4 KD, which is processed into a 143 amino acid mature NT-6 protein. Examination of the structure of NT-6 has revealed that it contains a hydrophobic domain at the N-terminus with the characteristics of a signal peptide, a pro-region containing basic motifs necessary for the proteolytic cleavage of the precursor protein, and all six conserved disulphide linked cysteine residues present in all other members of the neurotrophin family. A feature unique to NT-6 is the presence of 22 amino acid residues inserted between the second and third conserved cysteine domain. Northern blotting and in situ hybridization analysis of NT-6 gene expression has revealed that NT-6 mRNA is expressed in several teleost fish tissues including developing and adult brain and adult gill, liver, skin, heart, spleen, eye and muscle. (Gotz et al., 1994). NT-

6 has been shown to support the survival of NGF-dependent spinal sensory (DRG) and sympathetic neurons. However, NT-6 showed no survival effect on ciliary or BDNF-dependent nodose neurons, suggesting that the spectrum of responsive neurons is similar to that of NGF (Gotz et al., 1994).

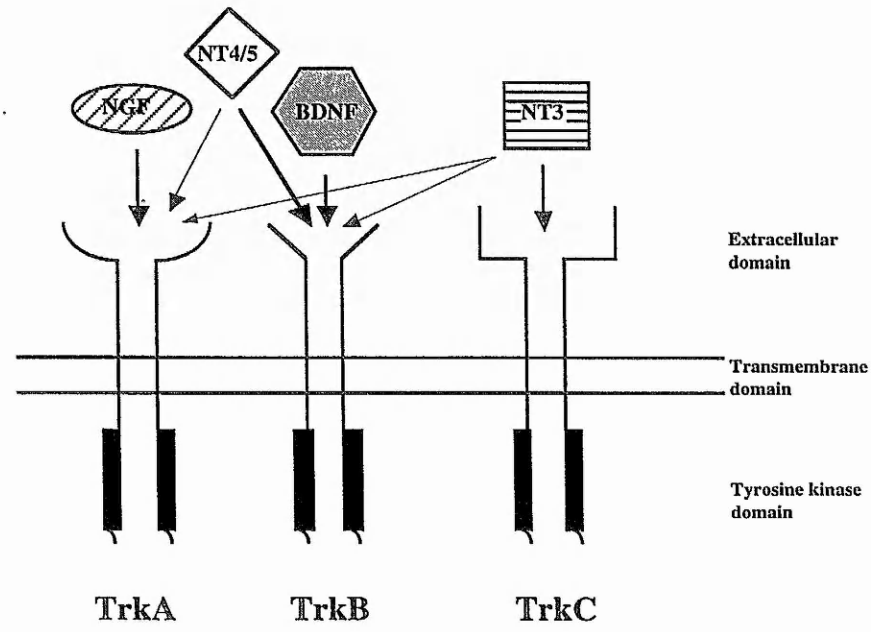
Neurotrophin 7

NT-7 is the most recent member of the neurotrophin family to be identified, and was isolated from the Carp *Cyprinus carpio* and Zebrafish *Danio rerio* using the polymerase chain reaction (PCR) (Nilsson et al., 1998; Ko et al., 1998). A characteristic feature of the neurotrophin structure is a conserved B turn of variable length flanked by two cysteine residues known as variable region III (McDonald et al., 1991). NT-7 contains a 15 aa residue insertion in a B-turn region in the middle of the mature protein, a position that corresponds to the insertion of 22 aa in NT-6. Apart from being rich in glycine and proline residues, the sequence in this region is unlike that of any other member of the neurotrophin family. However, amino acid sequence analysis of NT-7 has revealed a high degree of homology with fish NGF and NT-6 (65% and 63% respectively) (Nilsson et al., 1998; Ko et al., 1999). NT-7 supports the survival and neurite outgrowth of chicken DRG neurons. NT-7 interacts with the human p75 neurotrophin receptor and phosphorylation assays of Trk receptors in fibroblast cells have shown that NT-7 is able to induce tyrosine phosphorylation of rat TrkA (Nilsson et al., 1998; Ko et al., 1998).

1.4 Neurotrophin Receptors

The biological actions of members of the neurotrophin family are mediated through binding to two types of transmembrane receptors which possess different binding affinities (Meakin & Shooter, 1992; Barbacid, 1994). Low affinity binding is via p75, a transmembrane protein of 75KD, which contains a highly glycosylated

extracellular region rich in cysteine residues and a short cytoplasmic domain that is required for biological activity (Johnson et al., 1986; Radeke et al., 1987). The p75 neurotrophin receptor belongs to a family of transmembrane molecules including Fas and CD40 (Chao, 1994) which serve as receptors for the tumor necrosis factor family of cytokines. p75 binds to all members of the neurotrophin family with similar affinity with a dissociation constant of (10^{-8} to 10^{-9}M) (reviewed by Chao et al., 1986; Hempstead et al., 1991; Radeke et al., 1997). High affinity binding (10^{-10} to 10^{-11} M) of neurotrophins is mediated via the products of the trk proto-oncogenes (Cordon-Cardo et al., 1991; Hempstead et al., 1991; Kaplan et al., 1991a, 1991b; Klein et al., 1991a). TrkA binds to NGF and NT-3 (Cordon-Cardo et al., 1991; Hempstead et al., 1991; Kaplan et al., 1991a; Klein et al 1991a) whilst TrkB binds NT-4/5, BDNF and NT-3 (Berkemeier et al 1991; Klein et al 1992) and TrkC binds NT-3 (Lamballe et al 1991) see figure 1.1. Neurotrophin binding to Trk stimulates Trk autophosphorylation. This in turn activates a number of signalling pathways including those involving Ras and PI-3 kinase (Barbacid, 1994; Kaplan & Stephens, 1994, Greene & Kaplan, 1995; Kaplan & Miller, 1997).



Adapted From Davies, 1994b

Figure 1.1 Schematic diagram summarizing the interaction of neurotrophins with members of the Trk family of tyrosine kinase receptors.

The Low Affinity NGF receptor (p75)

The gene encoding the human p75 was first identified by gene transfer assays followed by immunological detection of transfected NGF receptor expressing cells (Chao et al., 1986). The p75 gene has since been cloned in rat and chicken (Johnson et al., 1986;Radeke et al., 1987; Large et al., 1989). The 222 amino acid long extracellular domain has four cysteine repeats characteristic of a loose family of cell surface receptors that includes; the lymphocyte surface antigen CD30 (Durkop et al., 1992), CD40 (Stamenkovic et al., 1989), OX40 (Mallet et al., 1990), the apoptosis-mediating fas cell surface antigen (Itoh et al., 1991), and the type I and II receptors for tumor necrosis factor (TNF) (Loetscher et al., 1990; Schall et al., 1990).

p75 is widely expressed in the PNS and CNS (Ernfors et al., 1988; Yan & Johnson, 1989). In-situ hybridization has revealed that in the PNS, p75 mRNA is expressed during development in NGF-dependent spinal and cranial sensory neurons, as well as sympathetic neurons (Heuer et al., 1990; Hallbook et al., 1990). In the CNS, p75 is expressed throughout brain development, with high levels being detected in the cerebellum and septum. p75 mRNA is present in a variety of non-neuronal cells of neuroectodermal origin including melanocytes, meningeal cells, glial cells and Schwann cells. Schwann cells secrete a proteolytically cleaved form of p75 of unknown function (Distefano & Johnson., 1988). Several other tissues also express P75 mRNA including basal keratinocytes, epithelial cells within the ducts and acini of mammary and prostatic glands and spleen cells (Thomson et al., 1988; Chesa et al., 1988).

Functional analysis has revealed that the functions of p75 are very diverse and complex. Kinetic studies have demonstrated that p75 is able to bind all neurotrophins with similar affinity, although the dissociation rate of NGF is greater than that of either NT-3 or BDNF (Rodriguez-Tebar et al., 1992). In vitro studies have demonstrated that NGF binding to p75 enhances the sensitivity of embryonic cranial sensory and postnatal sympathetic neurons to NGF at different stages of development (Horton et al., 1997; Ryden et al., 1997). Coexpression of TrkA or TrkB with p75 increases the binding affinity of TrkA for NGF and TrkB for BDNF (Makadeo et al., 1994; Bidel et al., 1999). Overexpression of p75 in MAH cells expressing TrkA increases NGF stimulated Trk autophosphorylation (Verdi et al., 1994), whilst inhibiting of NGF binding to p75 in PC12 cells reduces NGF mediated TrkA autophosphorylation (Barker et al. 1994). The p75 receptor plays a role in ligand discrimination by the Trk receptors. The ability of NT-3 to activate TrkA in the PC12 cells is enhanced when the binding of NT-3 to p75 is prevented by function-inhibiting antibodies, or when p75 expression is very low (Benedetti et al., 1993; Clary & Reichardt, 1994). Likewise, sympathetic neurons from postnatal p75-deficient mice are more sensitive to NT-3 than sympathetic neurons from wild-type mice, suggesting that p75 reduces the ability of NT-3 to signal via TrkA. Likewise, studies with mutated BDNF and NT4/5 proteins that bind TrkB normally but fail to bind p75 suggest that p75 also plays a role in TrkB ligand discrimination. Whereas the BDNF mutant activates TrkB as effectively as does wild-type BDNF, the NT4/5 mutant activates TrkB less effectively than wild-type NT4/5 (Ryden et al., 1995).

More recently, evidence has emerged to support the idea that under certain circumstances p75 can promote cell death. Increasing p75 expression in PC12 cells cultured without NGF stimulates increased cell death (Rabizadeh et al., 1993), whereas addition of anti-sense p75 oligonucleotides to postnatal DRG neurons in culture, promotes neuronal survival. In contrast anti-p75 oligonucleotides do not rescue

embryonic DRG neurons grown without NGF, rather they reduce the survival promoting effects of NGF, suggesting that the role of p75 undergoes a developmental switch from enhancing the survival-promoting actions of NGF during the fetal period to promoting neuronal death in the absence of a ligand postnatally (Barret et al., 1994). Recently, p75 has also been demonstrated to mediate cell death in a ligand-dependent fashion by mediating a cytotoxic response to neurotrophins in certain cell types and neurons (Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Davey & Davies 1998). In vitro studies have shown that NGF reduces the survival of chick TMN neurons cultured with CNTF, but not BDNF. The killing effect of NGF can be inhibited by anti-p75 antibodies, suggesting that NGF mediated cytotoxicity occurs via a p75 dependent mechanism (Davey & Davies 1998). Evidence for this new ligand dependent function of p75 has also been demonstrated in avian retina, mouse sympathetic ganglia and developing spinal cord of mice (Frade et al., 1996; Frade & Barde, 1998, Bamji et al., 1998; Frade & Barde, 1999).

Mice containing a homozygous null mutation for the p75 gene are viable and fertile (Lee et al., 1994). p75 deficient mice display neuronal loss in populations of sensory and sympathetic neurons in the PNS. In contrast the CNS exhibits no neuronal loss (Lee et al., 1994). Embryonic and postnatal trigeminal and dorsal root ganglion sensory neurons and postnatal sympathetic neurons from the superior cervical ganglia from p75-deficient mice show a decreased sensitivity to NGF, requiring higher concentrations of NGF for survival than heterozygous and wild-type neurons. This observation supports the hypothesis that p75 enhances the sensitivity of NGF-responsive neurons to NGF. In contrast, p75 deficient neurons do not show decreased sensitivity to other members of the neurotrophin family (Davies et al., 1993b; Lee et al., 1994). Immunohistochemical studies have shown that the foot pads of mutant mice are characterized by reduced sensory innervation by NGF dependent calcitonin gene-related peptide (CGRP) and substance P-immunoreactive nociceptive

neurons. This loss of nociceptive innervation accounts for the decreased response to thermal stimuli exhibited by p75 deficient mice, in addition to the observed ulceration of the extremities (Lee et al., 1994).

TrkA

Trk was first identified as a result of an oncogenic transformation caused by the chromosomal translocation of tropomyosin sequences onto the catalytic domain of TrkA in a human colon carcinoma (Martin-Zanca et al., 1986, 1989). TrkA has subsequently been identified as a signal transducing receptor for NGF (Kaplan et al., 1991a,b; Klein et al., 1991a). The human Trk proto-oncogene encodes a 140 KD glycoprotein termed gp140 ^{trk} which is produced from a primary translated product of 110 KD. This 110 KD protein is processed and glycosylated during translocation within the endoplasmic reticulum to yield the mature gp 140 $^{proto\text{-}trk}$. The Trk protooncogene encodes two isoforms of the TrkA protein, one of 790 aa and the other of 796 aa (Martin-Zanca et al., 1989; Meakin et al., 1992; Barker et al., 1993). Both Trk isoforms possess a typical structure of a tyrosine kinase cell surface receptor including; a 32-amino acid long putative signal peptide, a NH2-terminal ligand binding extracellular domain rich in consensus sites for N-glycosylation, a single transmembrane domain, a cytoplasmic domain containing a catalytic tyrosine kinase domain. However, the two isoforms differ by the presence or absence of a 6 amino acid insert situated in the extracellular domain close to the transmembrane domain. Studies have shown that the 790aa isoform is expressed predominantly in non-neuronal cells, whereas the isoform 796 is primarily expressed in neurons (Barker et al., 1993). NGF was shown to activate each form of the receptor comparably, however the 760 aa isoform showed binding and response to NT-3 (Clary & Reichardt, 1994). In vitro studies have also demonstrated that TrkA mediates NT-3 and NT-4/5 signalling (Berkemeier et al., 1991; Davies et al., 1995). TrkA mRNA is expressed by numerous

neurons within the PNS, including dorsal root, trigeminal, superior and jugular and sympathetic ganglia (Martin-Zanca et al 1990). In the CNS, TrkA mRNA is expressed in the septum, striatum and brainstem as well as in cholinergic neurons in the basal forebrain and neostriatum (Ringstedt et al., 1993; Holtzman et al., 1995) and non-cholinergic neurons in the paraventricular anterior and thalamic nuclei (Holtzman et al., 1995). TrkA mRNA is also found in non-neuronal tissue such as certain activated CD4+ T lymphocytes and human monocytes (Ehrhard et al., 1993).

Mice that contain a homozygous null mutation for the TrkA gene are viable at birth, but they do not gain weight in comparison to their littermates and die within the first month of life (Smeyne et al., 1994; Crowley et al., 1994). TrkA deficient mice also exhibit deficits in sensitivity to pain, temperature and noxious olfactory stimuli as well as exhibiting extensive sensory and sympathetic neuropathies (Crowley et al., 1994). Extensive neuronal cell loss occurs in trigeminal, sympathetic and dorsal root ganglia of TrkA deficient mice. In the CNS, TrkA deficient mice show a reduction in the cholinergic basal forebrain projections to the hippocampus and cerebral cortex (Smeyne et al., 1994).

TrkB

TrkB was isolated during analysis of Trk-related sequences from mouse brain cDNA libraries (Klein et al., 1989). Mouse TrkB shows a high degree of sequence homology to the human Trk proto-oncogene, with both genes exhibiting 57% homology within the extracellular domain, including 9 of the 11 cysteines present within the trk proto-oncogene, and 88% homology within their tyrosine kinase catalytic domains (Klein et al., 1989). TrkB locus is synthesized as a 821 amino acid precursor protein which undergoes post-translational glycosylation to give a glycoprotein of 145 KD (Klein et al., 1989; 1990; 1991; Middlemas et al., 1991). In the mouse, the TrkB locus exhibits a highly complex transcriptional pattern producing at least eight different

RNA transcripts, ranging in size from 0.7 to 9 kb which encode isoforms that possess ($TrkB^{TK+}$) or lack ($TrkB^{TK-}$) the tyrosine kinase domain (Klein et al., 1989; 1990a; Middlemas et al., 991). Two isoforms of the non-signal transducing $TrkB^{TK-}$ have been identified, both of which contain a very short cytoplasmic domain and lack the entire tyrosine kinase catalytic region (Klein et al., 1990a; Middlemas et al., 1991). Two novel variants of TrkB have been isolated that are both generated via alternative splicing and lack either two or all three of the leucine rich motifs. In the extracellular domain, both $TrkB^{TK-}$ and $TrkB^{TK+}$ isoforms of these leucine motif deleted variants exist (Ninkina et al., 1997). Expression studies using fibroblast cell lines which stably express these variants show that they do not bind to any of the TrkB ligands, suggesting that the leucine rich motifs in TrkB are essential for ligand binding and signalling (Ninkina et al., 1997). The pattern of TrkB mRNA expression in the developing trigeminal ganglia raises the possibility that BDNF responsiveness in developing sensory neurons is modulated by the relative level of catalytic and non-catalytic TrkB isoforms. This is supported by the results of microinjection studies that show that overexpressing TrkB $TrkB^{TK-}$ isoforms decreases the sensitivity to BDNF (Ninkina et al., 1996). Functional studies have shown that TrkB binds BDNF, amphibian NT-4 and mammalian NT-4/5, inducing autophosphorylation of TrkB (Berkemeier et al., 1991; Klein et al., 1992). In addition, studies have also demonstrated that NT-3 binds and signals via TrkB (Davies et al., 1995; Rarinas et al., 1998; Haung et al., 1999). Northern blotting and in situ hybridization studies have revealed that TrkB is widely expressed in the CNS and PNS (Klein et al., 1990a,b) including brain, spinal cord, spinal and cranial sensory ganglia and paravertebral sympathetic ganglia (Klein et al., 1989; Ringstedt et al., 1993). Northern blotting has revealed that in adult mice TrkB is preferentially expressed in the brain and the spinal cord, although significant levels of TrkB RNA have also been shown in non-neuronal tissue, such as lung, muscle and ovaries (Klein et al., 1989).

Mice with a targeted mutation in the TrkB gene which prevents expression of the $TrkB^{TK+}$ signalling receptor but not of the non-catalytic $TrkB^{TK-}$ isoforms are viable at birth but are unable to feed and die within the first 48hrs of life (Klein et al., 1993). Histological studies of the PNS of TrkB deficient mice revealed severe neuronal loss in the trigeminal and dorsal root ganglia compared to their wild type littermates (Klein et al., 1993). Within the CNS, neuronal loss was observed in TrkB null mutant mice in the facial motor nucleus and in the spinal cord at lumbar levels L2-L5 (Klein et al., 1993).

TrkC

TrkC was identified following the screening of a porcine brain cDNA library with a probe derived from the catalytic domain of the human trk proto-oncogene (Lamballa et al., 1991). The product of the TrkC gene is a 145 KD glycoprotein termed $gp145^{trkc}$ that shares a high degree of homology to other members of the Trk family. To date, four $TrkC^{TK+}$ and four $TrkC^{TK-}$ receptor isoforms have been identified (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner & large, 1994). The $TrkC^{TK+}$ isoforms, TrkC K1, K14, K25 and K39, differ in the number of additional amino acid residues located after the conserved sequence motif in the kinase domain which contains the putative autophosphorylation sites. The $TrkC^{TK-}$ isoforms have the same extracellular and transmembrane domain as the TrkC tyrosine kinases, but lack the kinase domain, and have been designated TrkC- K158, K143, K113 and K108 (Tsoulfas et al., 1993; Valenzuela et al., 1993). Functional studies utilizing cell lines ectopically expressing TrkC have demonstrated that TrkC is a receptor tyrosine kinase for NT-3 (Lamballe et al., 1991). In situ hybridization and Northern blot analysis have shown that TrkC transcripts are preferentially expressed within the CNS, with transcripts most notably in the hippocampus, cerebral cortex, and granular cell layer of the cerebellum (Lamballa et al., 1991). Within the PNS, TrkC has

been detected within various cranial ganglia (VII, VIII and X) as well as the trigeminal and dorsal root ganglia (Tessarollo et al., 1993; Lamballe et al., 1994). TrkC is also expressed within the myenteric plexi which form the ganglia of the enteric nervous system (Tessarollo et al., 1993; Lamballe et al., 1994). TrkC transcripts have also been detected within non-neuronal tissue including dental papillae, tongue, sublingual glands and the olfactory epithelium (Tessarollo et al., 1993; Lamballe et al., 1994). Structures within the body cavity expressing TrkC include the mesenchyme surrounding mesophenic and urogenital ducts as well as the diaphragm and adrenal gland (Tessarollo et al., 1993; Lamballe et al., 1994).

Mice with a targeted deletion of the TrkC kinase domain are viable at birth and display no visible defects at birth. However, these mice develop at a reduced rate and die within the first month of life (Klein et al., 1994). TrkC-deficient mice show a marked depletion of large myelinated group 1a axons which innervate muscle spindles in the periphery and which extend the collateral 1a projections to the spinal cord and synapse with spinocerebellar neurons. These defects are likely to be responsible for the abnormal movements characteristic of TrkC deficient mice (Klein et al., 1994). TrkC deficient mice also exhibit profound deficiencies within the peripheral nervous system with TrkC deficient mice exhibiting a reduction in vestibular neurons and a specific loss of cochlear neurons which innervate the inner hair cells (Schimmang et al., 1995). Spinal sensory ganglia also exhibit sensory neuron deficiencies, with lumbar DRG of TrkC deficient embryos exhibiting an 18% reduction in neuron numbers (Klein et al., 1994; Minichiello et al., 1995). However, neonatal NT-3 deficient mice exhibit a 66% reduction of lumbar DRG neurons (Ernfors et al., 1994a; Farinas et al 1994). The number of neurons in spiral ganglia of NT-3 deficient mice exhibit a reduction of 86%, whilst TrkC deficient mice exhibit a reduction of 51% (Schimmang et al., 1995). These differences may be due to the ability of NT-3 to signal via TrkA and TrkB receptors, as NT-3 has been shown to promote the in vitro survival of trigeminal and

nodose sensory neurons from TrkC deficient embryos, but not from TrkA or TrkB deficient embryos (Davies et al., 1995). Within the CNS, TrkC deficient mice exhibited no significant abnormalities even though TrkC is expressed throughout the CNS (Klein et al., 1994).

1.5 Other Neurotrophic Factors And Their Receptors

1.5.1 Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF) also known as hepatopoietin A or Scatter Factor (Miyazawa et al., 1989, Nakamura et., 1989, Zarnegar et al., 1989; Zarnger & Michalopoulos, 1989; Furlong et al., 1991; Naldini et al., 1991a; Weidner et al., 1991) was originally identified in the serum of partially hepatectomized rats as a potent mitogen for cultured rat hepatocytes (Nakamura et al., 1984, Michalopoulos et al., 1984). HGF has since been isolated from rat platelets (Nakamura et al., 1986) rabbit serum (Zarnegar & Michalopoulos, 1989) and from serum of human patients with fulminant hepatic failure or following a partial hepatectomy (Seldon et al., 1986; Gohda et al., 1988). HGF was purified from rat platelets and shown to be a heat and acid-labile cationic protein (Nakamura et al., 1986). Further characterization has revealed HGF to have a molecular weight of 82KD and to be a disulphide linked heterodimer ie a glycoprotein comprising a heavy alpha 69-55 KD subunit and a light beta 32-36 KD subunit (Nakamura et al., 1987, Godha et al ,1988; Zarnegar & Michalopoulos, 1989; Gherardi et al., 1989; Weider et al., 1990). Examination of the nucleotide sequence of human, mouse and rat HGF cDNA has shown that both the alpha and beta subunits are derived from a single open reading frame that encodes a 728 amino acid polypeptide precursor (Nakamura et al., 1989; Tashiro et al., 1990). The HGF alpha subunit is encoded between amino acids 55 to 494, whilst the beta subunit is encoded between amino acids 495 to 728. After biosynthesis, the alpha and

beta subunits are believed to be separated by a urokinase-type protease (Tashiro et al., 1990; Mizuno et al., 1992; Naldini et al., 1995). Mutation of critical amino acids within the cleavage site generates biologically inactive HGF proteins (Hartmann et al., 1992; Lokker et al., 1992), indicating that proteolytic cleavage is necessary for the production of biologically active HGF. The amino acid sequence of the rat HGF precursor shows considerable sequence homology (38%) to human plasminogen and other related proteases involved in blood coagulation. The HGF alpha subunit contains a hairpin loop of 27 amino acids at its amino terminus and four unique domains termed kringle domains, whilst plasminogen contains five kringle structures (Nakamura et al., 1989; Tashiro et al., 1990). The beta subunit of rat HGF shows a high degree of homology (37%) with various serine proteases, however, the histidine and serine residues of the protease active site are replaced in the beta chain of rat HGF by glutamine and tyrosine, respectively, resulting in HGF showing no proteolytic activity (Tashiro et al., 1990). A naturally occurring splice variant of HGF has been identified that lacks 5 amino acids within the 1st kringle domain. Functional analysis has suggested subtle differences in biological activities between the two isoforms (Seki et al., 1990; Shima et al ., 1991). Analysis of human genomic DNA showed that the multiple domains of the HGF protein are encoded as separate exons within the HGF gene (Miyazawa et al., 1991) which is localized on chromosome segment 7q 21.1 (Fukuyama et al., 1991; Weidner et al., 1991). The mouse HGF gene shows a high degree of homology to human HGF and is composed of 18 exons interrupted by 17 introns, spanning 65Kb (Liu et al., 1994).

HGF has been identified as the ligand for the proto-oncogene c-met, a membrane spanning tyrosine kinase receptor. This was initially demonstrated by immuno-coprecipitation of HGF/MET complexes and by chemical crosslinking of HGF to the beta subunit of the met protein. Upon binding of HGF, the met receptor undergoes autophosphorylation on two tyrosine residues, Try ^{1234} and Tyr ^{1235}

within the kinase domain, resulting in the upregulation of the met receptors kinase activity (Bottaro et al., 1991; Ferracini et al., 1991; Naldini et al., 1991a, 1991c; Hartmann et al., 1992). Chimeric receptors containing the cytoplasmic domain of the c-met receptor fused to the extracellular domain of a tyrosine kinase receptor have demonstrated that c-met is able to mediate the biological responses of HGF in different cell lines (Rong et al., 1992; Higuchi et al., 1992; Giordano et al., 1993; Komada et al., 1993; Weidner et al., 1993). Deletion studies have suggested that the binding of HGF to c-met is mediated via both the N-terminal hair pin containing region of HGF and the 1st kringle domain (Lokker et al., 1992; Okigaki et al., 1992). Two classes of HGF binding sites, have recently been identified on HGF responsive cells that differ in their binding affinity for HGF (Arahaki et al., 1992, Komada et al., 1992; Naldini et al., 1991b). The c-met receptor is responsible for high affinity binding, whilst low affinity binding is mediated by cell surface associated heparin-like molecules (Naldini et al., 1991 b). In mammals HGF mRNA is widely expressed in cells of mesenchymal origin such as lung, kidney, thymus, liver and gastrointestinal tract

(Stoker et al., 1987; Tashiro et al., 1990; Zarnegar et al., 1990; Defrances et al., 1992; Wang et al., 1994; Nakamura et al., 1995). HGF mRNA is expressed within developing and postnatal skeletal muscle as well as in skeletal muscle undergoing regeneration (Jennische et al., 1993). In addition, HGF mRNA is expressed in both the developing and adult mammalian central and peripheral nervous systems (Tashiro et al., 1990; Di Renzo et al., 1991; Komada et al., 1992; Thewke et al., 1996). In particular HGF mRNA expression is seen within neurons in the hippocampus, cortex and granule cell layer of the cerebellum, as well as in glial cells in the cingulum, cerebellum, pons and medulla regions of the brain (Jung et al., 1994; Honda et al., 1995; Yamagata et al., 1995). HGF expression has also been identified within

Henson's node, a region of the developing embryo which emits neuralizing signals (Streit et al., 1995).

HGF is a pleiotropic factor that exerts a variety of effects on many cell types during development and throughout life. HGF is a strong mitogen for hepatocytes (Nakamura et al., 1987; Gohda et al., 1988; Zarnegar & Michalopoulos 1989; Rubin et al., 1991), it is a motility and invasion inducing factor for epithelial and endothelial cells, and a potent angionic factor in vitro and in vivo (Stoker et al., 1987; Gherardi et al., 1989; Rosen et al., 1990; Weidner et al., 1990, 1991; Matsumoto et al., 1991; Bussolino et al., 1992). HGF also acts as a morphogen in vitro (Stern et al., 1990), inducing kidney epithelial cells to form branching ducts in collagen gels (Montesano et al., 1991) and triggering intrinsic morphogenic programs in a variety of epithelial cell lines (Brinkmann et al., 1995; Borros et al., 1995; Woolf et al., 1995; Soriano et al., 1995; Niranjan et al., 1995; Tabata et al.,1996; Matsubara et al., 1996). HGF stimulates the growth of other epithelial cells, such as kidney tubular epithelium, keratinocytes (Kan et al., 1991), endothelial cells and melanocytes (Igawa et al., 1991; Rubin et al., 1991). HGF can also modulate the motility, proliferation and proteoglycan rate of synthesis of chondrocytes (Takebayashi et al., 1995). In addition, HGF can also function as a renotrophic and pulmotrophic factor in the regeneration of kidney and lung respectively (Igawa et al., 1993; Nagaike et al., 1991; Yanagita et al., 1993), as well as being a hepatotrophic factor involved in liver regeneration (Ishiki et al., 1992; Ishibashi et al., 1992; Kawaida et al., 1994). HGF has also been shown to be involved in wound repair, with HGF stimulating early repair of rabbit esophageal epithelial cells following mucosal damage (Takahashi et al., 1995). HGF/Met signalling enhances tumor development, with high levels of HGF and c-met mRNA expression being shown in several tumor tissues (Rong et al., 1992; Di Renzo, 1991; Moriyama et al., 1995; Borset et al., 1996). Conversely, HGF also shows anti-proliferative activity in malignant cells (Shiota et al 1992, Tajima et al 1991) and

inhibits the growth of certain tumor cell lines, suggesting that HGF acts as a bi-directional growth regulator (Higashio et al., 1990; Conner et al., 1997).

There is growing evidence that HGF can function as an anti-apoptotic factor (Bardelli et al., 1996). HGF acts as a survival factor for PC12 cells and promotes the survival and differentiation of hepatocytes during development (Matsumoto et al., 1995; Schmidt et al., 1995). Within the developing central and peripheral nervous systems HGF has been shown to posses a wide spectrum of activities (Maina & Klein, 1999). For example, HGF has been shown to act as a muscle derived axonal chemoattractant and survival factor for motor neurons in vitro, as well as enhancing the neurotrophic actions of ciliary neurotrophic factor (CNTF) on motor neurons (Ebens et al., 1996, Wong et al., 1997). HGF also promotes neurite outgrowth from neocortical explants and enhances the number of tyrosine hydroxylase (TH)-positive neurons in vitro (Hamanoue et al., 1996). HGF is a potent mitogen for rat sciatic nerve Schwann cells (Krasnoselsky et al 1994).

In situ hybridization studies of various embryonic organs have identified transcripts of c-met transcripts in specific epithelial cells and expression of the HGF gene in cells of mesenchymal origin in adjacent tissue, suggesting a role for HGF/Met signalling in mesenchymal-epithelial interactions (Ishibashi et al., 1992; Honda et al., 1995; Kasai et al., 1996). During early embryonic development, both morphogenesis and differentiation of epithelial tissue are known to be influenced by mesenchymal-epithelial interactions, supporting the idea that HGF is involved in early embryonic development (Sonnenberg et al., 1993).

Experiments on mice with homozygous null mutations of the HGF and p190 ^{met} genes have facilitated our knowledge of the roles of HGF/Met signalling *in vivo*. Mice homozygous for HGF and c-met null mutations have indistinguishable phenotypes and show embryonic lethality between E12.5 and E15.5, as a result of abnormal liver development (Schmidt et al., 1995; Uehara et al., 1995). In mutant

embryos, the liver shows a significant reduction in size as well as extensive loss of parenchymal cells (Schmidt et al., 1995). Consistent with the scattering activity of HGF, met-deficient embryos show a failure of myogenic precursor cells to migrate, preventing normal development of limbs, diaphragm and tongue muscles (Bladt et al., 1995). Over expression of HGF in transgenic mice induces ectopic development of skeletal muscle and melanocytes in the central nervous system, supporting the idea that during development HGF acts in vivo as a morphogenic and epithelial scattering factor (Takayama et al 1996).

1.5.2 190^{met} Receptor

The HGF receptor is a transmembrane receptor encoded by the Met proto-oncogene ($p190^{met}$). The met proto-oncogene was originally identified as an activated oncogene in a human osteosarcoma cell line (Cooper et al., 1984). Activation of met involved chromosomal rearrangement and genetic fusion between two distinct genetic loci. Met, located on chromosome 7, and a translocated promoter region (tpr) located on chromosome 1 (Park et al., 1986). The tpr region of Tpr-Met encodes a leucine zipper motif which mediates dimerization of Tpr-Met (Rodrigues & Park, 1993). This in turn leads to constitutive phosphorylation and kinase activity of the Tpr-Met oncoprotein (Fixman et al., 1996). Oncogenic activity of the Tpr-Met oncoprotein is dependent upon phosphorylation of a tyrosine residue (Y^{489}) located within the carboxyl terminus which acts as a direct binding site for the Grb2 adaptor protein (Kamikura et al., 1996).

The structural and functional properties of the met proto-oncogene have demonstrated that it is a member of the receptor tyrosine kinase gene (RTK) family (Dean et al., 1985; Park et al., 1987). Structural analysis of the c-met gene product in a gastric carcinoma cell line (GTL-16) overexpressing the gene has revealed that the mature form of the receptor is a heterodimer of 190 KD consisting of two disulphide

linked chains of 50KD (p50α) and a 145 KD (p145β) (Giordano et al., 1989a). The receptor is initially synthesized as a single chain 170KD precursor (pr170), which undergoes glycosylation and proteolytic cleavage to generate the mature p190 ^{met} heterodimer (Giordano et al., 1989b). The extracellular ligand binding domain of met is derived from the NH2-terminal portion of the p145β-chain and the p50α chain. The cytoplasmic kinase domain is encoded by the carboxy terminal of p145β (Gonzatti-Haces et al., 1988, Park et al., 1987; Tempest et al., 1988). Examination of the nucleotide sequence of c-met DNA has revealed that the primary structure of c-met is highly conserved amongst different mammalian species (Liu et al., 1996). Isolation and characterization of human genomic DNA clones has shown that the c-met gene spans approximately 120 Kb in length and consists of 21 exons interrupted by 20 introns (Liu et al., 1998). Two isoforms of Met containing small deletions generated by alternative splicing have been described. One lacks 18 amino acids in the extracellular domain (Rodrigues et al., 1991) and the other lacks 47 amino acids in the intracytoplasmic juxtamembrane domain (Lee et al., 1995). Two additional isoforms of met have been identified, p140 ^{met} and p130 ^{met} (Prat et al., 1991). p140 ^{met} is membrane bound and contains an α chain and a carboxy-terminal truncated β chain of 85KD that lacks the cytoplasmic kinase domain. The second isoform p130 ^{met} is a soluble isoform that consists of an α chain and a 75KD carboxy-terminal truncated β chain (P75β) that lacks the kinase domain. Both truncated isoforms are generated by posttranslational proteolysis and are consistently detected together with p190 ^{met} in different cell lines with p140 ^{met} being present at half the amount of p190 ^{met} (Prat et al., 1991). Upon binding of HGF, the p190 ^{met} receptor undergoes autophosphorylation on tyrosine residues of the 145-KD β subunit (Bottaro et al., 1991, Naldini et al., 1991b, 1991c, Lokker et al., 1992). The kinase activity of p190 ^{met} is modulated by phosphorylation of regulatory sites. Negative modulation is mediated by phosphorylation of a serine residue (ser 985) located within the

juxtamembrane domain of the β chain. This residue is phosphorylated following activation of protein kinase C or a serine kinase sensitive to intracellular Ca^{2+} levels (Gandino et al., 1990; 1991, 1994). Positive regulation of p190 ^{Met} is mediated by ligand induced autophoshorylation of Tyr 489 (Naldini et al 1991c; Kamikuza et al., 1996).

The major autophosphorylation site has been identified as Tyr ^{1235} (Ferracini et al., 1991) and is located within the tyrosine kinase domain in a region homologous to major autophosphorylation sites in other receptor and non-receptor kinases (Hanks et al., 1988). In the p190 ^{met} kinase, Tyr ^{1235} is in close proximity to two other tyrosine residues, Tyr ^{1230} and Tyr ^{1234}. This 3 tyrosine motif is highly conserved amongst tyrosine kinase receptors for IGF-1 (Insulin Growth Factor-1) and NGF and in the products of the c-ros and sevenless genes (Hanks et al., 1988). The substitution of both Tyr ^{1234} and Tyr^{1235} by another amino acid using site directed mutagenesis produces a molecule which is unable to be activated by autophosphorylation (Longati et al., 1994; Ponzetto et al., 1994; Zhu et al., 1994; Fixman et al., 1995). In contrast, replacement of Tyr ^{1230} does not decrease the receptor's ability to autophosphorylate, showing that following ligand binding both Tyr ^{1234} and Tyr ^{1235} but not Tyr ^{1230} are essential for activation of the p190^{Met} kinase (Longati et al., 1994). Experiments using cDNA expression vectors encoding hybrid tyrosine kinase receptors containing the extracellular domain of a tyrosine kinase receptor and the membrane spanning and cytoplasmic domain of c-met, have shown that HGF induced activation of p190 ^{met} is sufficient to mediate HGF induced biological responses (Komada et al., 1993; Giordano et al., 1993; Weidner et al., 1993). Following autophosphorylation, the subsequent phosphorylation of tyrosine residues in the non-catalytic carboxy-terminal region of p190^{Met} produces multifunctional high affinity docking sites for effector proteins containing Src homology 2 domains (SH2 domains) (Graziani et al., 1991; Bardelli et al., 1992; Ponzetto, 1994). The majority of SH2 binding is mediated via a

single multifunctional docking site consisting of two phosphotyrosine residues ($Y^{1349}VHVNATY^{1356}VNV$). Mutations of Tyr^{1349} and Tyr^{1356} abrogates all in vitro and in vivo Met kinase activity (Ponzetto et al., 1994; Maina et al., 1996), including the recruitment of several signal transducers and adaptor molecules, such as Grb2, Gab1, SHC, phosphatidylinositol 3-kinase (PI3-kinase) and phospholipase-Cy (PLC-γ) (Ponzetto et al., 1994; Bardelli et al., 1992). Following docking to activated met receptors, SH2 domain containing proteins in turn activate a number of signalling pathways including the Ras/Map kinase and JNK/SAP kinase pathways via Grb2 and Gab1. In addition met activates phospholipid pathways through PLC-γ, phosphotyrosine-mediated pathways through interaction with src tyrosine kinase, the PI3-kinase pathways, SHP2 tyrosine phosphatase, Nck and the STAT pathways (Ponzetto et al., 1994; Kochhar et al., 1996; Weider et al., 1996; Nguyen et al., 1997; Rodrigues et al., 1997; Boccaccio et al., 1998).

Expression studies have shown that c-Met mRNA is widely expressed in a variety of mammalian tissues containing an epithelial component including: liver, stomach, gut, thyroid, kidney, lung, uterus, ovaries, testicular tissue, skin, prostate, intestinal tissue (Naldini et al., 1991a; Tashiro et al., 1990; Zarnegar et al., 1990; Sonnerberg et al., 1993; Di Renzo et al., 1993; Krasnoselsky et al., 1994; Honda et al., 1995; Andermacher et al., 1996; Depuydt et al., 1996). c-met expression has also been observed in various structures of the embryonic and postnatal nervous system including the brain, spinal cord, motor neurons and microglial cells (Sonnernberg et al., 1993; Jung et al., 1994; Andermarcher et al., 1996; Yamada et al., 1994; Honda et al., 1995). Examination of the subcellular localization of c-met in various epithelial tissues has shown that it is predominantly located at the basolateral plasma membrane of polarized epithelial cells (Crepaldi et al., 1994a).

1.5.3 Neurotrophic Cytokines

Ciliary neurotrophic factor (CNTF), Leukaemia inhibiting factor (LIF), cardiotrophin-1 (CT-1), oncostatin-M (OSM) and interleukin-6 (IL-6), are all members of a family of proteins, termed cytokines, which have been shown to have multiple actions on cells of the nervous system (Sendtner et al., 1994; Stahl & Yancopoulos, 1994; Pennica et al., 1995a; Horton et al., 1998). Although the different members of the family show limited sequence homology (15% at the a.a. level) they do share several structural characteristics (Bazan et al., 1991; Robinson et al., 1994; McDonald et al., 1995) and signal via oligomeric receptor complexes that have one or more common components (Stahl & Yancopoulos, 1994; Wollert et al., 1996).

Ciliary Neurotrophic Factor

CNTF was initially identified and purified from chicken eye tissue as a result of its ability to promote the survival of embryonic parasympathetic neurons from chicken ciliary ganglia (Alder et al., 1979; Barbin et al., 1984). CNTF was subsequently purified from adult rat sciatic nerve (Manthorpe et al., 1986). Based on amino acid sequence analysis the cDNAs for rabbit and rat CNTF have been cloned. More recently the mouse and human CNTF cDNAs have been identified and analyzed. In mouse the CNTF gene is located on chromosome 19 (Kaupmann et al., 1991) and in humans on chromosome 11 (Lam et al., 1991). Mature CNTF is a 200 amino acid acidic protein with a molecular weight of 20-24 KD (Lin et al., 1989; Stockli et al., 1989). Within the CNS and PNS, CNTF has been shown to support the survival of several neuronal populations in vitro. In the PNS, CNTF supports the survival of parasympathetic neurons within the embryonic chick ciliary ganglion, sympathetic neurons of the lumbar sympathetic ganglia and sensory neurons of the nodose, trigeminal and dorsal root ganglia (Barbin et al., 1984; Manthorpe & Varon, 1985; Arakawa et al 1990; Oppenheim et al ., 1991; Martinou et al., 1992; Vejsada et al., 1995; Burnham et al.,

1994; Horton et al., 1998). Overexpression of CNTF in vivo has been shown to rescue chick ciliary ganglion neurons from cell death (Finn et al., 1998). Within the CNS, CNTF is involved in promoting the survival of adult motoneurons in vivo (Masu et al., 1993), and can promote the survival of hippocampal neurons (Ip et al., 1991) and purkinje cells in vitro (Larkfors et al., 1994). CNTF also prevents the axotomy-induced neuronal degeneration of a variety of neurons including facial (Sendtner et al., 1990) and spinal motor neurons (Li et al., 1994; Vejsada et al., 1995), as well as neurons within the thalamic nuclei and dopaminergic neurons of the substantia nigra (Clatterbuck et al., 1993; Hagg & Varon, 1993). CNTF mRNA cannot be detected by Northern blotting in tissue of newborn rats (Stockli et al., 1989) and significant amounts of CNTF mRNA are not expressed until the fourth week postnatally (Dobrea et al., 1992). In adult rats, CNTF mRNA is expressed at high levels in a variety of neuronal tissues including the optic nerve, sciatic nerve (Williams et al., 1984) olfactory bulb (Stockli et al., 1989) and spinal cord (Ip et al., 1993a). Low, but still significant amounts of CNTF mRNA are expressed within the brain stem, cerebellum, hippocampus and the midbrain (Stockli et al., 1991; Ip et al 1993a). Studies have shown that CNTF mRNA expression is significantly up-regulated following lesions of the hippocampus and cortex, with the maximum level reached after 3 days, and is sustained for up to 20 days. This suggests that CNTF may have a role in reponse to trauma (Ip et al., 1993b). Mice homozygous for a CNTF null mutation are viable at birth and initially thrive. Later in adult life CNTF null mice exhibit a mild progressive loss of motor neurons, resulting in minor muscle weakness (Masu et al 1993).

LIF, CT-1, OSM & IL-6

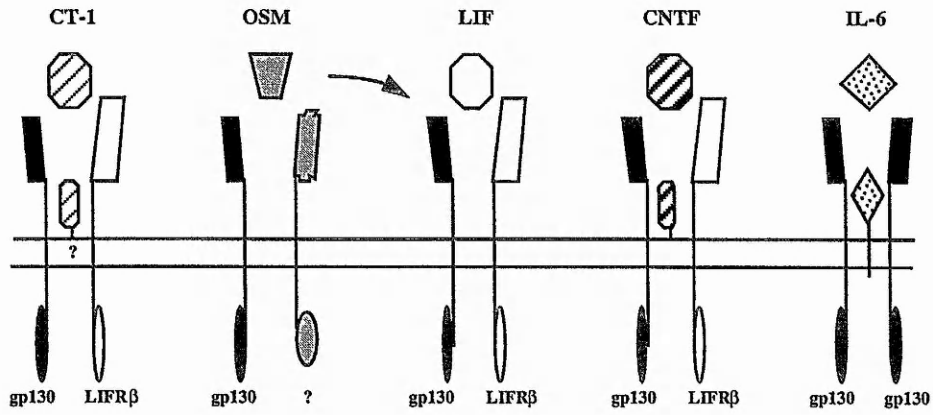
Other cytokines including LIF, CT-1, OSM and IL-6 have all been shown to promote the survival of various populations of CNS and PNS neurons. LIF has been shown to promote the survival of motoneurons, sympathetic neurons and certain

sensory neurons in vitro (Martinou et al., 1992; Murphy et al., 1993a; Kotzbauer et al 1994; Thaler et al., 1994; Horton et al., 1998). LIF also plays a role in promoting the survival of fetal motoneurons in vivo (Li et al., 1995; Sendtner et al., 1996). *In vitro* and *in vivo* studies have shown that LIF plays a role in determining cholinergic phenotype, particularly in sympathetic neurons (Ure et al., 1992; Bamber et al., 1994). LIF has been shown to promote the differentiation of oligodendrocyte-type-2-astrocytes (0-2A) progenitors derived from the optic nerve into both oligodendrocytes and astrocytes (Mayer et al., 1994). CT-1 promotes the survival of cultured ciliary ganglion neurons (Pennica et al., 1995b), midbrain dopaminergic neurons and motor neurons (Pennica et al., 1995b, 1996) and embryonic and postnatal sensory neurons (Horton et al., 1998, Hall et al., 1999). IL-6 enhances the in vitro survival of a proportion of forebrain cholinergic and midbrain catecholaminergic neurons and embryonic sensory neurons (Hama et al., 1989; Kushima et al., 1992; Horton et al., 1998), whilst OSM promotes the survival of a subset of late fetal DRG neurons in culture, as well as embryonic sensory neurons of the trigeminal and nodose ganglia (Ware et al., 1995, Horton et al., 1998).

1.5.4 Cytokine Receptors

The neurotrophic cytokines signal via a multicomponent receptor signalling system (Fig 1.2). All cytokine receptors consist of dimers of their signal transducing β components, consisting of either gp130 homodimers or gp130/LIFRβ heterodimers. Only receptors for CNTF and Il-6 have an additional α component. Specific receptor subunits determine the specificity for each cytokine. CNTFRα and the LIFRβ / gp130 heterodimer are specific receptors for CNTF, LIFRβ / gp130 heterdimers are specific for LIF (Ip et al 1992,) IL-6Rα and the gp130 / gp130 homodimer are specific for IL-6 (Bruce et al., 1992), whilst in the case of OSM an additional OSM specific component may replace LIFRβ (Davis & Yancopoulo, 1993; Stahl & Yancopoulo,

1994). The CNTF α receptor lacks transmembrane and cytoplasmic domains, and is anchored to the cell surface via a glycosyl phosphatidylinositol (GPI) linkage (Bazan et al., 1991; Davies et al., 1991; Ip et al., 1992b; Stahl & Yancopoulos, 1994). CNTFα shows a high degree of sequence homology with IL-6Rα. IL-6Rα is not able to initiate signalling, but after binding to IL-6 it can bind to gp130, to initiate signalling (Taga et al., 1989; Hibi et al., 1990; Kishimoto et al., 1992).



Adapted from Stahl and Yancopoulos, 1994

Figure 1.2 Schematic diagram showing the interaction of neurotrophic cytokines their receptors.

The first step in the formation of the CNTF receptor involves the binding of the CNTF ligand to its CNTFRα component, followed by recruitment of the β components to form the complete receptor complex. The β components do not bind to CNTF in the absence of CNTFRα (Ip et al., 1992b; Davis et al., 1993). In CNTF-responsive cells, the binding of CNTF to it receptor induces tyrosine phosphorylation of gp130 and LIFRβ initiating subsequent signalling activation (Stahl & Yancopoulos, 1993, 1994). The specific receptor subunits utilized by each cytokine explains the cell-type specificity of the cytokines whilst explaining why certain cytokines appear to have identical

actions on some targets. In particular, the neural specificity of CNTF is due to the restricted expression of the CNTFRα and LIFRβ subunits. In contrast, the variety of actions of LIF are due to the widespread distribution gp130 and LIFRβ. Following ligand binding, cytokine signalling is mediated via the activation of Janus kinases Jak 1, Jak 2 and transcription factors of the STAT family, in particular STAT1 and STAT3, and the tyrosine phosphatase SHP2 [SH2 (src homology 2) domain-containing tyrosine phosphatase] (Stahl & Yancopoulos, 1994).

1.5.5 The TGFβ Family of Neurotrophic Factors and Their Receptors.

The transforming growth factor β (TGFβ) family of proteins consist of a number of structurally related growth factors that regulate cell growth, differentiation, motility and cell death during development (Massague, 1994). Recent studies have identified a subfamily of the TGFβ family consisting of four members and termed the GDNF family, which have been shown to act as potent survival factors for cultured PNS and CNS neurons. The GDNF family consists of glial cell line-derived neurotrophic factor (GDNF) (Linet al., 1993), neurturin (NTN) (Kotzbauer et al., 1996), persephin (PSP) (Milbrandt et al., 1998), and artemin (ARTN) (Baloh et al., 1998b).

GDNF and Neurturin both support the in vitro survival of rat sympathetic (Kotzbauer et al., 1996), midbrain dopaminergic (Lin et al., 1993; Horger et al., 1998), dorsal root ganglion (DRG) (Buj-Bello et al., 1995; Molliver et al., 1997), nodose ganglion (Kotzbauer et al., 1996) and enteric neurons (Hearn et al., 1998; Heuckeroth et al., 1998). Neurturin also plays a role in the development and maintenance of the enteric nervous system in vivo (Heuckeroth et al., 1999). Artemin supports sensory, sympathetic and dopaminergic midbrain neurons in culture (Baloh et al., 1998b). Persephin promotes the survival of motoneurons but does not support PNS neurons (Heuckeroth et al., 1998; Milbrandt et al., 1998). Biochemical

studies have shown that GDNF ligands mediate their activities through a family of multicomponent receptors that are composed of the transmembrane tyrosine kinase Ret and one of four glycosyl-phosphatidyl inositol- (GPI-) linked proteins termed GFRα1-GFRα4 (Jing et al., 1996; Treanor et al., 1996; Baloh et al., 1997; 1998a; Buj-Bello et al., 1997; Klein et al., 1997; Widenfalk et al., 1997; Naveilhan et al., 1998; Nomoto et al., 1998; Thompson et al., 1998; Trupp et al., 1998; Worby et al., 1998). In vitro studies have shown that GFRα1 is the preferred coreceptor for GDNF (Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997). GFRa2 is the preferred coreceptor for NTN, GFRα3 is the preferred coreceptor for artemin (Baloh et al., 1998a) and GFRα4 is the preferred coreceptor for PSP (Enokido et al., 1998). Certain members of the GDNF ligand family are able to use alternate GFRα-Ret complexes at higher ligand concentrations. For example, NTN and artemin can activate Ret via GFRα1 in vitro, whereas GDNF can activate Ret via GFRα2.

1.5.6 Objectives Of This Study

I) To examine the *in vitro effect* of HGF/met signaling on neurite outgrowth and survival in embryonic sensory neurons of the mouse dorsal root ganglia during development.

II) To examine the *in vivo* and *in vitro* effect of HGF/Met signalling on several different developmental processess in neural-crest derived sympathetic neurons at different stages during embryonic development.

III) To examine the *in vitro effect* of HGF/met signaling on neurite outgrowth and survival in chicken embryonic parasympathetic and proprioceptive neurons during development.

IV) To examine the influence of the anti-apoptotic cytoplasmic protein Bcl-2 on regulating axonal growth rates in embryonic sensory neurons from the trigeminal ganglia using Bcl-2- deficient mice.

Chapter 2
Material and Methods

2.1 Preparation of the culture substratum

Neurons were grown on a laminin/polyornithine substratum. For most purposes, 35mm or 60mm diameter Nunc (Gibco) plastic tissue culture petri dishes were used. 1ml of 0.5mg/ml polyornithine (P-8638, Sigma) in a 0.15M borate buffer (pH 8.6) was placed in to each dish and left to stand overnight at room temperature. The poly-DL-ornithine solution was filter sterilized using a 0.2μm filter and stored in glass bottles at 4^0C for up to two weeks. The polyornithine solution was aspirated the following morning and the dishes were washed three times with sterile distilled water. The dishes were then left to air dry in a laminar flow hood for 20 minutes. 150 μl of a 20 μg/ml solution of laminin in F14 medium was placed in the centre of the each dish and using the pipette tip the laminin was spread over two thirds of the dish surface. The dishes were then placed in a 4% $C0_2$ incubator for at least 4 hours. Laminin (Gibco) is available as a sterile solution which is thawed at 4°C, aliquoted in to sterile Eppendorf tubes and stored at -70°C until required. The required number of aliquots were thawed at 4°C and the laminin was diluted in F14 immediately before use. Once the laminined dishes were removed from the incubator they were washed twice with F 12+ 10% HIHS medium. The surfaces of the dishes were not allowed to dry between washes and 1ml of F14+Sato additives for mouse cultures and 1ml of F14+10% HIHS medium for chicken cultures, was placed in each dish after washing.

2.2 Preparation of culture medium

Powdered F14 (a special formulation from Imperial labs) was made up with highly purified water that was sequentially passed through a charcoal filter, reverse osmosis system and a Milli-Q system before being double distilled. The F14 medium was made up from a frozen x10 concentrate (stored in 50ml aliquots kept

at -40°C). The x1 F14 was made up by adding 1g of sodium bicarbonate and a 50ml aliquot of x10 F14 to 450ml of water. CO_2 was bubbled through this medium until the pH reached pH7. The medium was then filter sterilised through a 0.2 μm filter, and stored at 4°C. For embryonic mouse neurons the following supplements; collectively termed Sato additives were added to the F14 medium: 2 mM glutamine, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite and 340 ng/ml tri-iodo-thyronine. The use of the Sato supplement was employed as it was not conducive for the growth of non-neuronal cells.

2.3 Dissection techniques

Instruments and Equipment

1. All dissections and preparations of neuronal cultures were carried out in a in a laminar flow hood using standard aseptic techniques.
2. Dissections involved using straight and curved fine watch makers forceps and tungsten needles.
3. Tungsten needles were made by immersing 3-5cm of bent 0.5mm diameter tungsten wire in 5M KOH with a 3-12 V-AC current passing through the wire and an electrode immersed in the solution to produce a fine needle.
4. All dissections were carried out in 65 mm petri dishes containing L15 medium that had previously been warmed to 37°C.
5. For the final steps of dissection, a good quality stereomicroscope with zoom lens capability was used. Illumination was provided by a fibre optic light source.

2.4 Dissection of Mouse Ganglia

Dissection of embryonic mouse sensory and sympathetic ganglia

Embryos were removed from pregnant females under sterile conditions. The pregnant females were killed at the required stage of gestation by cervical dislocation and 70% alcohol was then sprayed onto the abdomen of the pregnant females to sterilize the area. A small incision was made in the skin of the abdomen and the skin was then pulled away from the incision, tearing it and exposing the abdominal muscles beneath. Holding the anterior abdominal muscle with a pair of toothed forceps a small incision was made with a pair of fine scissors, taking care not to cut into the intestines. Once air had entered the peritoneal cavity through this hole, the incision could easily be extended exposing the embryonic mice. The uterine horns were removed from the pelvic cavity using a pair of fine scissors. The uterine wall, chorion and amnion were then removed from the embryos using watchmaker's forceps.

Dissection of E11 and E12 trigeminal ganglia

Using tungsten needles, two coronal incisions through the head were made, one just above each eye, the other between the maxillary and mandibular processes of the first branchial arch. The trigeminal ganglia could be seen as two opaque structures in the tissue slice obtained and could be easily removed from the tissue slice and freed of any adherent mesenchymal tissue using tungsten needles. To free the ganglion of this tissue, one needle was used to steady the ganglion and the other to pinch off the adherent tissue against the bottom of the Petri dish. The dissected ganglia were then transfered to a 35 mm Petri dish containing pre-heated L15 medium using a siliconised Pasteur pipette.

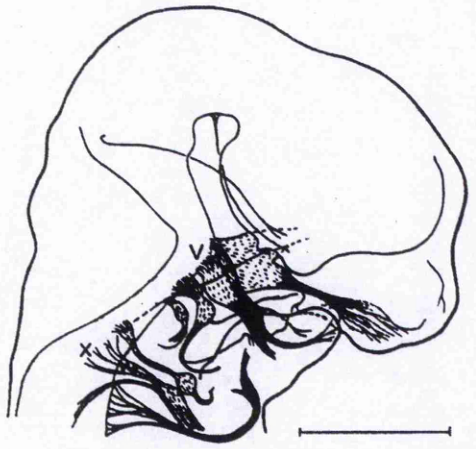


Figure 2.1: Drawing showing the medial aspect of the head of an E11 mouse embryo. The location of the trigeminal (V) and nodose (X) ganglia are marked. Scale bar = 1mm. (From Davies and Lumsden, 1987).

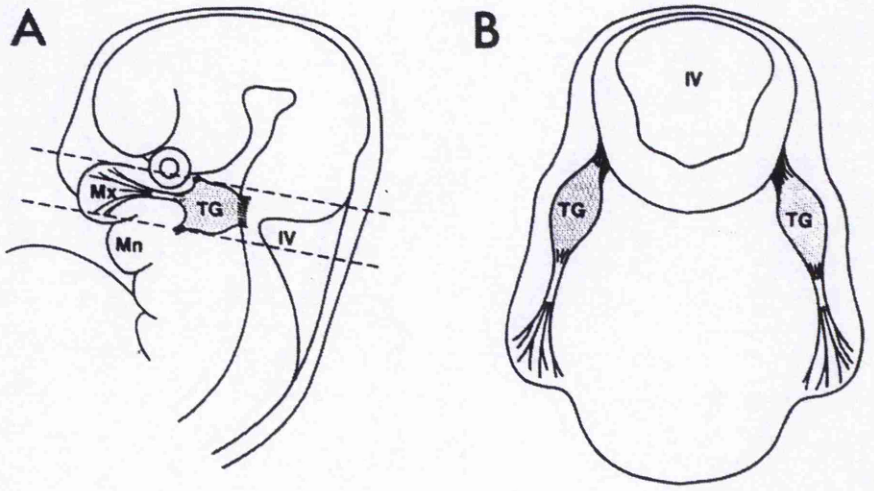


Figure 2.2: Drawings showing the dissection of the trigeminal ganglia from an E11 mouse embryo. **A**, lateral aspect of the E11 head showing the location of the transverse incision (interrupted lines) for obtaining a slice of tissue that contains both trigeminal ganglia. **B**, rostral aspect of this slice showing the location of the trigeminal ganglia. Trigeminal ganglion (TG), maxillary process (Mx), mandibular process (Mn), fourth ventricle (IV). (Adapted from Davies and Lumsden, 1984).

Dissection of mouse superior cervical sympathetic ganglia

In mouse embryos, the (SCG) are situated close to the base of the skull in close proximity to the nodose ganglia. The top of the skull and underlying forebrain were removed from the embryo using the same plane of section described for the first incision of the trigeminal dissection. The embryos were decapitated and the head was cut in half along the saggital plane. Using tungsten needles, the hindbrain was removed from each bissected head. The cleft-like jugular foramen was then opened to the midline by inserting one tungsten needle into the jugular foramen so that it lay beneath the base of the skull, medial to the foramen, and bringing a second needle into apposition with the first, so as to cut through the intervening tissues. Once the jugular foramen was opened, the SCG and the nodose ganglion were cleary visible lying at the base of the foramen. The nodose ganglion is spherical in structure with a prominent vagus nerve attached to its distal aspect. This ganglion is clearly distinguised from the SCG which is an elongated structure that is attached caudally to the sympathetic chain (which is much thinner than the vagus nerve) and is situated anterior to the internal carotid artery.

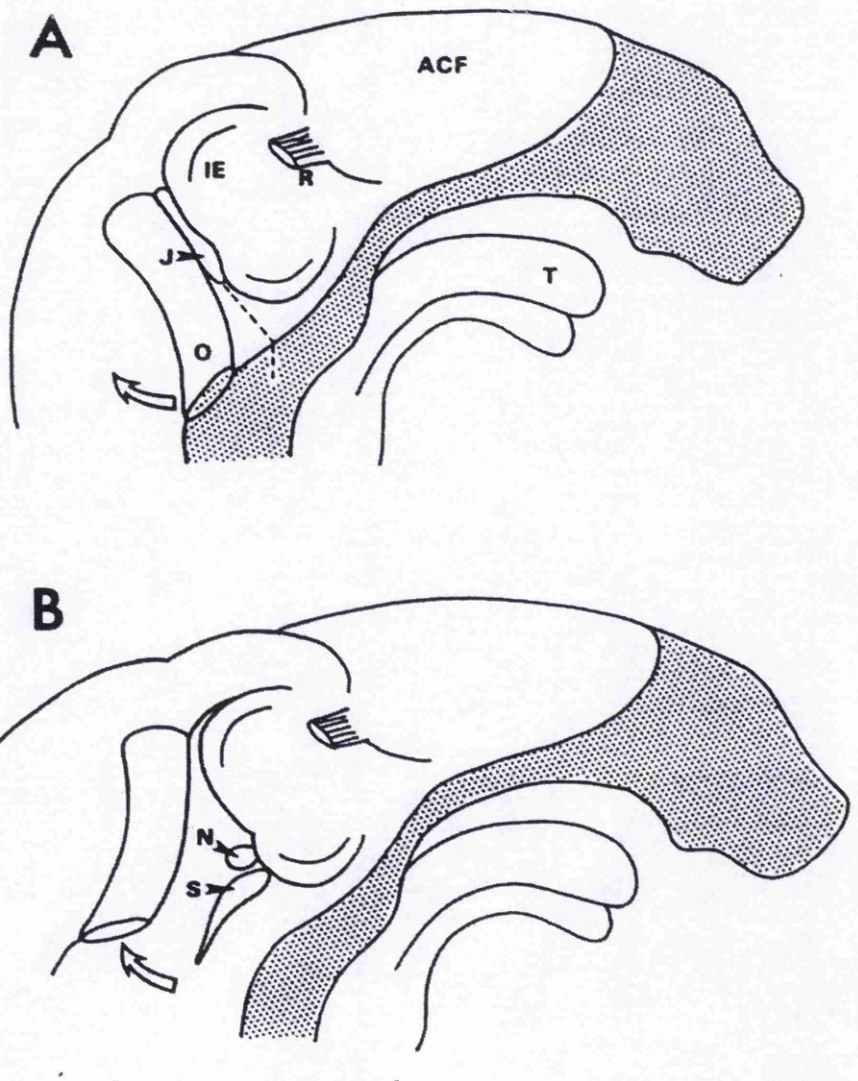


Figure 2.3: Drawings of the medial aspect of the left half of an E14 mouse embryo head showing successive stages in the dissection of the SCG. The bissected midline structures lying along and in front of the cranial base are stippled. A. The incision passing from the jugular foramen (J) to the midline is shown by the interrupted line. The direction in which the occipital bone (O) should be reflected to open up the jugular foramen after making the previous incision is shown by the large curved arrow. T, tongue; ACF, anterior cranial fossa; R, root of the trigeminal nerve; IE, inner ear. B. The nodose ganglion (N) and superior cervical sympathetic ganglion (S) are revealed after extending the jugular foramen to the midline and reflecting the large ossified part of the occipital bone backwards. (Scale bar = 1mm).

Dissection of paravertebral sympathetic chain ganglia

These ganglia are located either side of the midline of the thorax and abdomen lying lateral to the vertebral column and ventral to the dorsal root ganglia. A pair of curved watchmaker's forceps was used to remove the thoracic and abdominal viscera, exposing the verebral column and dorsal root ganglia. The posterior thoracic and abdominal walls were gently washed with L15 from a Pasteur pipette. Using a pair of watchmakers forceps, the ganglia were gently peeled away from the vertebral column and separated from non-neuronal connective tissue. Care was taken to ensure the ganglia were kept intact. The ganglia were then removed using a siliconised Pasteur pipette and pooled in a fresh 35mm Petri dish containing pre-heated L15 medium.

Dissection of Dorsal Root Ganglia

The DRG are situated in two parallel rows on either side of the vertebral column. The head, tail and limbs were removed leaving the thoracic and abdominal region. The ventral and lateral parts of the thoracic and abdominal walls were removed, leaving the dorsal surface of the embryo which contained the visibly distinguishable DRG on either side of the spinal cord. The DRG were then removed, and cleaned with tungsten needles removing all non-neuronal connective tissue.

2.4.1 Dissection of Chicken Ganglia

Dissection of chicken trigeminal mesencephalic nucleus (TMN)

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator until the desired age. After removing the chicken embryos from the eggs, the brains were collected in pre-heated L15 medium (40 to 60 in most preparations). The brains were then transfered to a fresh plastic petri dish containing L15, and the dissection was completed with tungsten needles as shown in Figure 2.4. The TMN

is located close to the posterior commissure overlying the cerebral aqueduct within the midbrain of developing chicken brains. The TMN was isolated by two coronal incisions in the tectal vesicle of the midbrain followed by removal of the ventral pia mater.

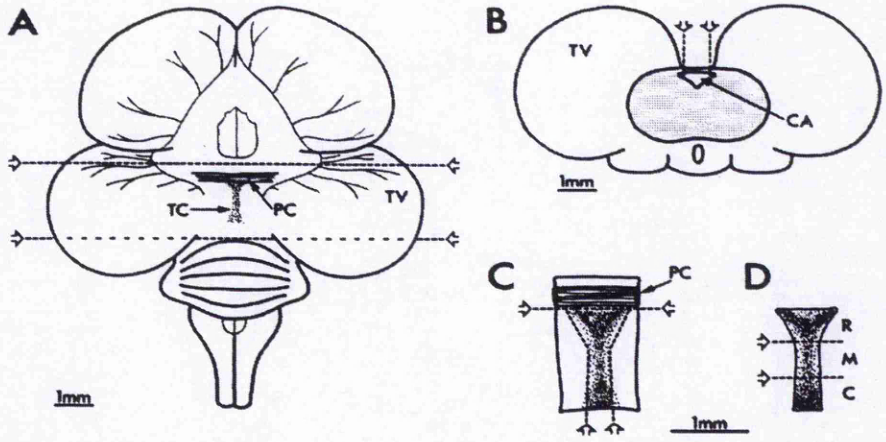


Figure 2.4: Successive stages in the dissection of the median part of the TMN from an E12 chick embryo. A, Dorsal aspect of the brain showing the location of the two coronal incissions (interrupted lines) for isolating the midbrain. B, Caudal aspect of the isolated midbrain showing the location of the two parasagittal incissions for removing the roof of the cerebral aqueduct after carefully stripping off the overlying pia mater. C, Dorsal aspect of the cerebral aqueduct showing the location of the incisions for cutting out the median part of the TMN. D, Subdissection of the median part of the TMN. Abbreviations; tectal vesicle (TV), tectal commissure (TC), posterior commissure (PC), cerebral aqueduct (CA) rostral (R), middle (M), caudal (C). (Adapted from Davies, 1986)

Dissection of chick ciliary ganglia

The parasympathetic neurons of the ciliary ganglia are located on the medial side of optic nerve on the posterior surface of the eye-ball. Chicken embryos were decapitated close to the base of the skull. Using curved forceps, the eye-ball was gently removed from the eye socket by scoring around the edge of the socket, (care was taken not to damage the eye or the underlying tissue within the socket). The ciliary ganglia were removed from the eyeball using fine watchmakers forceps. Using tungsten needles, non-neuronal connective tissue was removed from the

ciliary ganglia, which were then transfered using a siliconised pasteur pipette and pooled in to a 35mm Petri dish containing L15 medium.

2.5 Tissue dissociation techniques

Dissected neural tissue was incubated with trypsin, washed and triturated to give a single cell suspension. The procedure was similar for all embryonic mouse and chicken tissue, but the strength of the trypsin and incubation times were adjusted for the particular tissue. Using a Pasteur pipette, the dissected ganglia were transferred to a 10 ml conical tube containing 1 ml of calcium/magnesium-free Hanks Balanced Salt Solution (CMF-HBSS). The ganglia were washed, by agitation and the CMF-HBSS was then removed. For early mouse ganglia, 0.95 ml of fresh CMF-HBSS and 0.05 ml of 1% trypsin (Worthington) were added. The lower end of the tube was then immersed in a water bath at 37°C for between 5 and 10 minutes depending on the ganglia being dissociated. These times were adjusted for different batches of trypsin. The optimum time was discovered to be a compromise between neuronal damage due to over-trypsinization and neuronal damage due to the vigorous trituration required to dissociate under-trypsinized tissue. If the tissue started to disaggregate before trituration, the time was too long. If the tissue dissociated with difficulty and incompletely with trituration, then the time was not long enough. After trypsinization, most of the trypsin solution was removed and the embryonic tissue was washed with 2 x 10 ml of Hams F12 or F14 medium containing 10% heat-activated horse serum to remove and inactivate the residual trypsin. Removal of the medium was facilitated by pelleting the tissue between washings in a benchtop centrifuge, at 2000 x g, for 1 to 2 min. After washing, the tissue was dissociated into a single-cell suspension by trituration. Trituration was carried out using a siliconized Pasteur pipette, the tip of which had previously been flame polished using a bunsen burner flame to form a fine bore. The tissue was triturated in 1.5 ml of culture medium or CMF-HBSS. Trituration was performed by taking the tissue and medium up into the polished Pasteur

pippette, holding the pipette against the bottom of the tube and slowly expelling the contents with firm pressure. If done correctly, the early ganglionic tissue was completely dissociated after three to five passages. When using older tissue, some connective tissue fragments were left after all of the neurons had dissociated. These larger tissue fragments were allowed to settle after the first two or three passages. The trituration was monitored using an inverted phase contrast microscope, by examining a drop of the dissociated cell suspension on a glass slide. One characteristic of over-trituration is the loss of neuronal processes; neurons that have been triturated carefully should have long processes attached to their cell bodies.

2.5.1 Separation of neurons from non-neuronal cells

A variety of different non-neuronal cells synthesize and release neurotrophic factors in culture. It is therefore desirable to remove these cells prior to culture when studying the effects of neurotrophic factors on any population of neurons. If non-neuronal cells are not removed, the percentage neuronal survival in control cultures may be unacceptably high. Furthermore, if non-neuronal cells are present, it cannot be concluded whether the effect of a factor or reagent on neuronal survival is due to a direct action of the factor on the neurons, or whether it is mediated via the non-neuronal cells. Neuronal and non-neuronal cells were seperated using a technique called differential sedimentation. This technique was only used in experiments using chicken neurons, as chicken neurons are significantly larger than chicken non-neuronal cells. Early mouse neurons are similar in size to mouse non-neuronal cells and cannot be separated by this technique. In the case of mouse cultures, the use of the defined Sato medium, which does not support the survival of non-neuronal cells, reduced the number of non-neuronal cells.

2.5.2 Differential sedimentation of embryonic chicken neurons

This technique depends on differences in sedimentation rates of cells in a liquid medium. Generally, the larger the cell, the faster its rate of sedimentation, therefore neurons sediment more quickly than non-neuronal cells because of their greater size. Several variations of the method described by Miller and Phillips (1969) have been applied to embryonic neural tissue (Lam, 1972; Barkley, et al., 1973; Cohen, et al., 1973; Cohen et al; 1978; Berg & Fischbach, 1978). This method is applicable to all populations of neurons in the peripheral nervous system and is very efficient (at least 60% of the neurons in the starting tissue are recovered uncontaminated by other cells). Furthermore, since the cells sediment through culture medium, neuronal viability is very high.

Sedimentation was carried out in a 100 ml cylindrical, siliconised glass dropping funnel with a ground glass outlet tap (Figure 2.5). Funnels were sterilised before use by autoclaving. Each funnel was filled to a height of 8 to 10 cm with F14 medium plus 10% heat-inactivated horse serum (thoroughly mixed and filtered through a 0.2 µm millipore filter beforehand). Funnels were clamped vertically in a stand and placed on a vibration-free surface, at a temperature of 2°C, and left overnight with foil coverings. The dissociated cell suspension was usually made up to a volume of about 2 ml in HBSS. The cell suspension was carefully layered on the medium, by running it down the inside wall of the funnel, and the foil on top of the dropping funnel was then replaced. After an hour, the foil was removed from the spout and 4 to 5 ml aliquots were run off into sterile tubes. 0.5 ml samples of each fraction were taken and placed in a 24 multi-well plate (with 16 mm diameter wells). These were examined with a phase contrast microscope to determine which fractions contained only neurons. Mid-embryonic and older neurons were clearly distinguished from other cells by their characteristic rounded phase bright cell bodies and elongated processes. All of the neuronal fractions were pooled and were plated in Nunc tissue culture dishes, as described below.

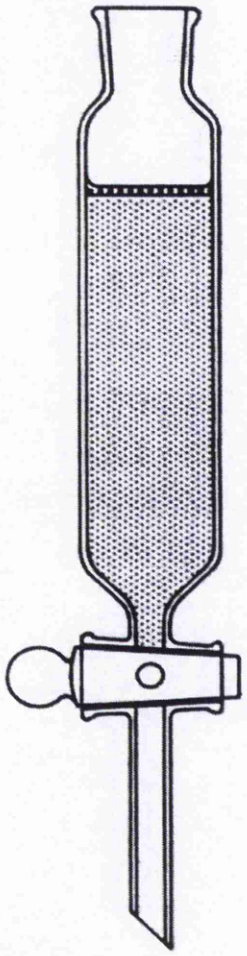


Figure 2.5: Drawing of a dropping funnel showing a cell suspension (heavy stipple) layered on culture medium (light stipple). (Scale bar= 2 cm).

2.5.3 Seeding the neurons

For cohort studies the most reliable results were obtained when the neurons were plated at a density of between 20 and 40 per dish. The suspension of neurons was placed in a suitable volume of medium in a large screw-top tube (eg 50 ml centrifuge tube) and the neurons were evenly distributed in the medium by gently rocking the tube end-over-end several times. The neuronal suspension was dispensed to each dish using a 1ml pipette, in two lots of 0.5 ml (to ensure even distribution). It was important to avoid touching the medium in the dishes with the pipette tip, otherwise small quantities of neurotrophic factor could be transferred to other dishes. Dishes were returned to a humidified CO_2 incubator at 37°C (5% CO_2 was used for F14 medium). Because of the very low neuron density required, the most reliable method of estimating the correct neuron density at the time of plating neurons was observation by eye. A 1ml aliquot of the cell suspension was placed in a test culture dish and, the resulting neuronal suspension was examined with a phase contrast microscope.

2.5.4 Quantification of neuronal survival

For this, a standard graticule for examining the same area of each culture dish was used. These were made from the base of a 90 mm plastic petri dish. A scalpel blade was used to inscribe a 6x6 grid of 2 mm^2 squares in the center of the dish. This graticule was mounted on the stage of an inverted phase contrast microscope with the inscribed surface uppermost. To determine the number of neurons seeded per dish, several dishes were examined after 6 hours in culture. Each dish was centred over the graticule, and the number of neurons within the inscribed area was counted. For the initial survival count, all phase bright attached cells were counted. A small percentage of neurons (usually less than 10%) are damaged during dissociation, and do not attach to the substratum, these neurons were ignored and only those that had attached were counted. The number of surviving neurons in all dishes was counted after a further 24 or 48 hrs in culture. The survival responses

of neurons to a particular neurotrophic factor are expressed as percentage survival; the number of surviving neurons expressed as a percentage of the initial number of neurons in the 12x12 mm grid.

2.5.5 Cohort Studies

In some experiments the survival of individual neurons, as opposed to the neuronal population as a whole, was determined. This was done by following the fate of individual neurons within an initial cohort of neurons over a set period of time, identified after 6 hours of plating. The position of individual identified neurons was marked by reference to 144 mm^2 grid. In some experiments, neurons that differentiated in culture from progenitors were also identified at set time points and their fate followed during further periods in culture. The fates of all the neurons in each cohort is expressed as a percentage of the size of the initial cohort identified at 6 hrs. To avoid any effects of neurotrophins on the size of the initial cohort, neurotrophins were added after the identification of the cohort at 6 hours.

2.5.6 Axonal Growth rate

To examine axonal growth rates, accurate, serial, camera lucida drawings of the same individual neurons over a set period were made with the aid of a drawing tube. Neurons were cultured in 60mm^2 diameter polyornithine /laminin coated dishes, with a 6x6mm square grid scored on the undersurface. Neurons were plated at a density of 20-40 neurons per dish. To exclude any differences in axonal growth rate that could arise as a consequence of differences in neuronal viability, only neurons that survived throughout the culture period were include in the analyses. Between 20-40 neurons were drawn and analysed in each cohort. The total axon length was determined by tracing the drawings onto a digitising pad linked to a computer containing an NIH Image program

2.6 Genotyping

Genotyping of Bcl-2 and met $^{D/D}$ knockout mice was done using a polymerase chain reaction (PCR). The PCR technique is a method for the enzymatic amplification of specific DNA sequences. The PCR method employed for genotyping used three synthetic oligonucleotide primers to amplify the wildtype and mutant alleles. One primer was common to both alleles, one was unique to the wild-type allele and one primer was unique to the mutant allele. The PCR cycle consists of three steps: Strand separation, primer hybridization and extension of primers by DNA synthesis, all of which are driven by changes in temperature. All primers were ordered from Gibco.

To determine the genotype of mice used in transgenic experiments a sample of tail or embryonic tissue was collected and digested over night in 500µl of Proteinase K solution at 55°C. The next day, 210µl of 5MNaCl was added, and following thorough mixing, the sample was spun for 10min at 13000g to pellet debri and precipitated protein. Next, 400µl of the supernatant, which contained the DNA, was added to 400µl of cold 100% ethanol, which resulted in the production of a cloudy DNA precipitate. The DNA was pelleted for 5 minutes at 13000g and the ethanol was aspirated with a 1ml micropipette. The DNA pellet was washed with 70% ethanol, before being allowed to air dry. The dried pellet was resuspended in 400-100µl of distilled water.

(i). Bcl-2 Mice

For genotyping Bcl-2, a separate 0.5ml microfuge tube for each DNA sample contained a 20.0 µl reaction volume, made up as follows:

1.2 µl	25mM $MgCl_2$
2 µl	10 x Taq buffer (Buffer B, Promega)
1µl	5mM dNTPs (Pharmacia)
0.2 µl	Primer cDNA3 (20µM)

0.1µl	Primer pGKNM (20µM)
0.216 µl	Primer cDNA5 (20µM)
0.5 µ1	Taq-thermostable DNA polymerase (Promega)
1 µl	template DNA
14µl	dd H_20

Following addition of the genomic DNA to the PCR reagents, the two solutions were mixed and overlaid with 40µl of mineral oil in order to prevent evaporation of the reactants within the tube. The solutions were then transfered to the heating block of a PCR machine where they were subjected to a sequence of 33 repeat cycles of denaturing annealing and elongation. Each PCR reaction was repeated twice to ensure accurate genotypes were obtained.

Bcl-2 PCR Program

Step 1: 94°C-5minute

Step 2: 94°C-1minute

Step 3: 64°C-1minute

Step 4: 72°C-1minute 30 seconds

Step 5: 72°C-10minutes

4°C soak

Steps 1 and 2; Strand separation. Step 3; Annealing. Steps 4 and 5; DNA synthesis. The cycle was repeated 33 times.

The primer sequences were as follows:

pGKNM 5' -GCC TAC CCG CTT CCA TTG CTC AGC 3'

cDNA5 5' -GCC CAG ACT CAT TCA ACC AGA CAT 3'

cDNA3 5' -CGT CCC TCT TCA TCA CCT TTC AGC 3'

Knockout and wildtype bands were 400bp and 900bp respectively.

The PCR products were seperated by electrophoresis on 2% agarose gels.

(ii). Met $^{d/d}$ Mice

For genotyping met$^{d/d}$ embryos a separate 0.5ml microfuge tube for each DNA sample contained the following.

1.2 µl	25mM MgCl2
2 µl	10x Taq buffer (Buffer B, promega)
1µl	5mM dNTPs
0.16µl	Primer 610 sense
0.64µl	Primer 611 antisense
0.16µl	Primer 655 sense
0.5µl	Taq Polymerase DNA polymerase (Promega)
13.5 µl	dd H_20
1µl	template DNA

Following addition of the genomic DNA to the PCR reagents, the two solutions were mixed and overlaid with 40µl of mineral oil in order to prevent evaporation of the reactants within the tube. The solutions were then transfered to the heating block of the PCR machine where they were subjected to a sequence of 33 repeat cycles of denaturing, annealing and elongation. Each PCR reaction was repeated twice to ensure accurate genotypes were obtained.

Met $^{d/d}$ PCR Program

Step 1: 94°C 5minutes

Step 2: 94°C 1minute

Step 3: 56°C 1minute

Step 4: 72°C 1minute 30 seconds

Step 5: 72°C 10minutes

4°C minutes soak

The cycle was repeated 33 times.

The primers used were:

610 sense	5' -AGGATTGATCATTGGTGCGGTC 3'
611 anti-sense	5' -CATCTCTGTAGTTGGACTTACAC 3'
655 sense	5' -CAGCTCATTCCTCCCACTC 3'

Knockout and wildtype bands were 550bp and 390bp respectively.

The PCR products were then seperated by electrophoresis on 2% agarose gels.

Separation of amplified DNA fragments was carried out using electrophoresis through agarose gels. This process relies on the principle that negatively charged DNA will migrate from the cathode to the anode when a potential difference is applied across the gel. Tris-acetate (TAE) electrophoresis buffer was used for agarose gel electrophoresis. TAE was prepared as a 50x stock solution consisting of 242grams of Tris base, 57.1 ml glacial acetic acid and 100ml 0.5M EDTA (pH8.0) diluted in 1 litre of deionized water. 4 grams of ultrapure nuclease free agarose (Gibco BRL) was added to 200ml of 1xTEA and melted in a microwave oven. The intercalating dye, ethidium bromide was added to both the molten agarose gel at a concentration of 0.5µg/ml. Once set, the gel was placed in an electrophoresis tank filled with TAE buffer and the well forming combs were removed. DNA samples, which were mixed with with the appropriate volume of 6x gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in deionized water) were then loaded into the wells of the gel. To determine the molecular size of the DNA fragments, DNA size markers containing DNA fragments of known lengths were also loaded onto the gel for comparison. A

voltage of 200v was applied across the gel for 30 minutes, after which DNA bands were viewed under U.V. light.

Solutions and Media

HBSS

Hank's balanced salt solution without calcium and magnesium (GibcoBRL).

0.1% Trypsin

50 mg of trypsin (Worthington) was added to 5ml of Ca/Mg free PBS (GibcoBRL) and sterilised with an 0.22µm filter (Gelman Sc.).

Poly-DL-ornithine

O.5 mg/ml poly-DL-ornithine (Sigma) in 0.15M boric acid (4.6 grams boric acid, BDH, in 500ml distilled water, pH 8.4) was sterilised with an 0.22µm filter (Gelman Sc.).

Laminin

Natural mouse laminin (Gibco) was stored at -70°C at a concentration of 1.08 mg/ml, in aliquotes of 20µl. 20 ml of concentrated laminin stock was diluted in 1ml of Ham's F-14 medium.

L-15

A 1L unit of L-15 powder (GibcoBRL0 wa added to 1L of double distilled water containing 100 mg streptomycin (Sigma) and 60 mg penicillin (Sigma), pH 7.3, and sterilised with an 0.22µm filter (Gelman Sc.).

Ham's F-12

A 1L unit of F12 powder (GibcoBRL) was added to 1L of double distilled water containing 100 mg streptomycin (Sigma) and 60 mg penicillin (Sigma), pH 7.3, and sterilised with an 0.22μm filter (Gelman Sc.).

Ham's F-14

10 x concentration stock solution:

A 10L unit of F14 powder (GibcoBRL) was added to 1L of double distilled water containing 500 mg streptomycin (Sigma) and 300 mg penicillin (Sigma).

The 10X stock was stored in 50 ml aliquots at -40°C.

1x working solution of F14:

A 50 ml 10x aliquot was diluted into 450 ml double distilled water. 1 gram of sodium hydrogen carbonate ($NaHC0_3$) (BDH) was added and the solution was adusted to pH 7 with dry ice and sterilised with an 0.22μm filter (Gelman Sc.).

HIHS

Heat-inactivated horse serum (Gibco).

Neurotrophic Factors

Recombinant human NGF was supplied by Genentech.

Recombinant rat BDNF was supplied by Regeneron Pharmaceuticals.

Recombinant rat NT-3 was supplied by Regeneron Pharmaceuticals.

Recombinant rat CNTF was supplied by Arnon Rosenthal

Polyclonal anti-HGF antibodies were supplied by R & D Systems.

Neurotrophins Factors were kept in F-14 plus 10% HIHS, pH5.5, at -80°C until use.

CHAPTER 3
The role of HGF/MET signalling in Sensory neuron development

3.1 Introduction

HGF is a pleiotropic factor that exerts its effects by binding to the Met receptor tyrosine kinase (Gherardi & Stoker 1991; Brinkmann et al., 1995; Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Maina et al., 1996). Prior to carrying out the work described in the Chapter, there were several published observations which suggested that HGF and Met signalling play a role in neural development. First, Met expression had been observed in various structures of the embryonic and postnatal nervous system, including spinal cord motoneurons and migrating neural crest cells (Sonnenberg et al., 1993; Jung et al., 1994; Andermarcher et al., 1996). Second, HGF had been shown to be a chemoattractant and survival factor for spinal motor neurons in vitro and HGF deficient mice showed marked deficits in motor fibres (Ebens et al., 1996). To further investigate the potential role of HGF in neural development, I carried out a series of in vitro investigations of the effect of HGF on the differentiation, survival and growth of sensory neurons of embryonic mouse dorsal root ganglia (DRG).

Sensory neurons within DRG are a heterogenous population specialized for conveying different modalities of sensory information. Different subpopulations of neurons innervate separate types of peripheral sensory end organs and each has a distinct axonal arboriation in the spinal cord. Recent studies using gene knockouts have shown that different subpopulations of DRG neurons depend on specific neurotrophins for their survival, with small nociceptive DRG neurons being NGF dependent (Crowley et al., 1994; Smeyne et al., 1994), large proprioceptive DRG neurons being dependent on NT-3 (Ernfors et al., 1994, Farinas et al., 1994; Klein et al., 1994), and a subpopulation of DRG neurons which may include

mechanoreceptors, are dependant on BDNF (Klein et al., 1993; Jones et al., 1994). Some studies also suggest that certain sensory neurons may be sensitive to more than one neurotrophin (Davies et al., 1986; Gaese et al., 1994).

The studies reported in this Chapter on the effects of HGF on cultured DRG neurons has demonstrated that HGF cooperates with NGF but not other neurotrophins to enhance differentiation, neuronal survival and axonal outgrowth.

3.2 Results

3.2.1 HGF cooperates with NGF to enhance sensory neuron differentiation and survival

To determine if HGF exhibits neurotrophic activity for DRG neurons, DRG were dissected from E12 mouse embryos, dissociated and cultured in defined serum free medium, containing either HGF alone or with HGF in a combination with the neurotrophins NGF, BDNF or NT-3. Defined cohorts of cells were identified at 6 hours and the fate of the cells was monitored at regular intervals over 72 hrs in culture (Fig. 3.1). HGF (5ng/ml) alone did not enhance neuronal survival compared to control cultures. This showed that HGF alone does not exhibit neurotrophic activity on DRG sensory neurons. However, when neurons were cultured with HGF and NGF, both at 5ng/ml, there were substantially more neurons surviving in culture compared to cultures containing NGF alone (Fig. 3.2). The cohort experiments in which the fate of individual cells were followed over a set time period, indicated that the increase in neuron survival was the result of two effects of HGF. First in the presence of NGF, HGF increased the length of time that differentiated neurons survive. Secondly, HGF in combination with NGF also increased the number of neurons that differentiated from progenitor cells in culture. In contrast, HGF did not increase the survival of DRG sensory neurons treated with either BDNF (Fig. 3.3) or NT-3 (Fig. 3.4) at a concentration of 5ng/ml. HGF also did not increase the number of neuron differentiating from progenitors in cultures containing BDNF or NT-3 at these concentrations. HGF failed to enhance the effects of higher concentration of BDNF (50 ng/ml) data not shown. However experiments with higher concentrations of NT3 were not done because NT3 is known to cross-reactive with the NGF receptor TrkA, that would almost certainly lead to stimulation of NGF dependent neurons (Klein et al., 1991; Davies et al., 1995).

3.2.2 HGF cooperates with NGF to enhance axonal outgrowth from cultured DRG neurons

To investigate whether HGF influences the growth of axons emanating from developing DRG neurons, I studied the growth rate of axons in dissociated DRG neurons cultured in NGF (10 ng/ml) alone or in combination with HGF (5ng/ml or 25ng/ml) (Fig.3.5). The axonal growth rate of individual E12 DRG neurons was significantly enhanced in the presence of HGF plus NGF, compared to NGF alone ($p < 0.005$; t tests), demonstrating that HGF enhances the axonal growth rates of neurons surviving with NGF. Due to the fact that the results were obtained from neurons grown at very low densities, these results further indicate that HGF exerts a direct effect on NGF dependent neurons rather than acting indirectly on other cells. The typical appearances of E12 DRG neurons grown for 72 hrs with NGF plus HGF and NGF alone are shown in figure 3.6.

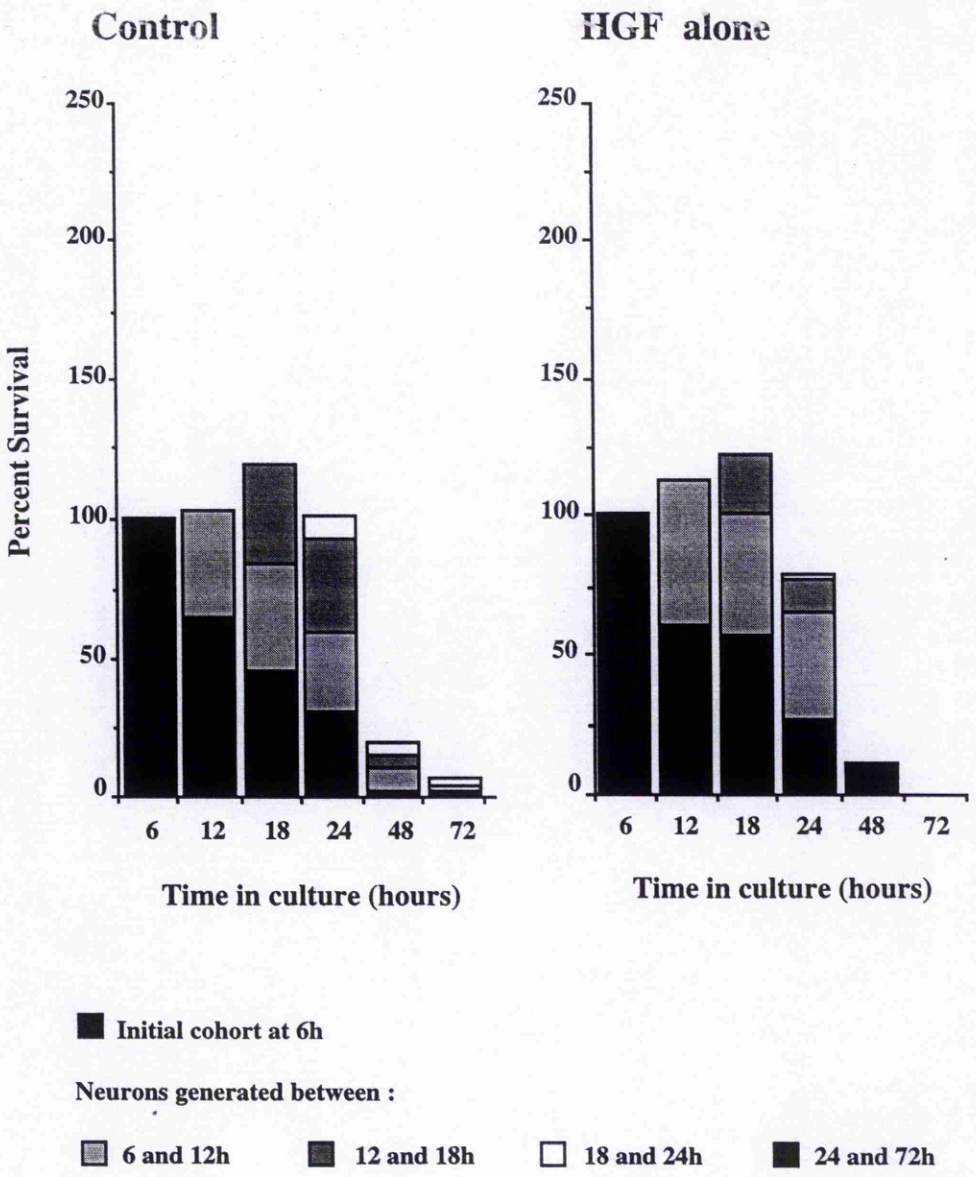


Figure 3.1: Bar charts showing the results of cohort experiments to determine the survival of thoracic DRG neurons that differentiate after different periods in culture. Neurons were grown either without factors (control) or with 5 ng/ml of HGF. Similar results were obtained in three separate experiments.

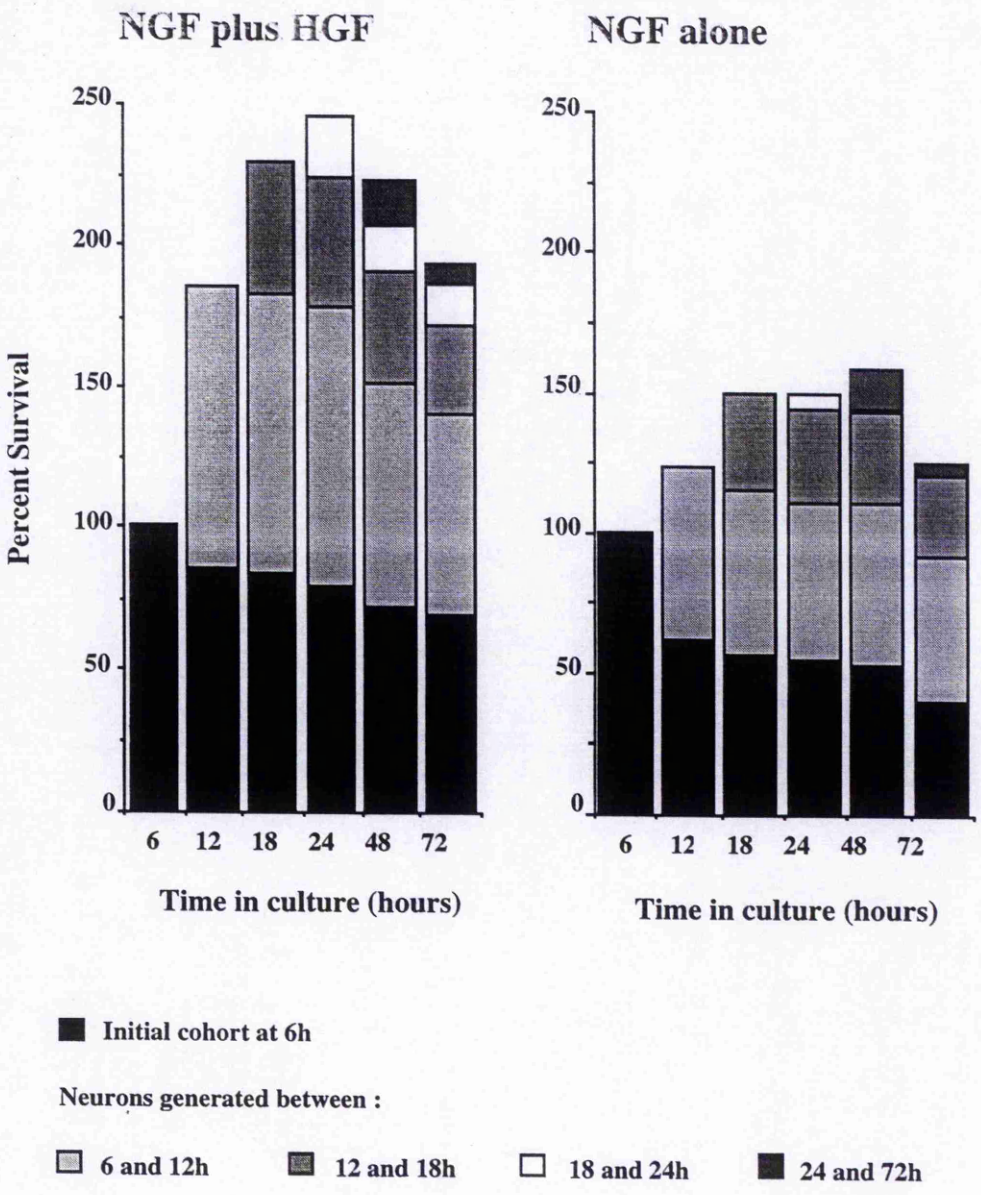


Figure 3.2: Bar charts showing the results of cohort experiments to determine the survival of thoracic DRG neurons that differentiate after different periods in culture. Neurons were grown either with 5 ng/ml of NGF or NGF plus 5ng/ml HGF. Similar results were obtained in three separate experiments.

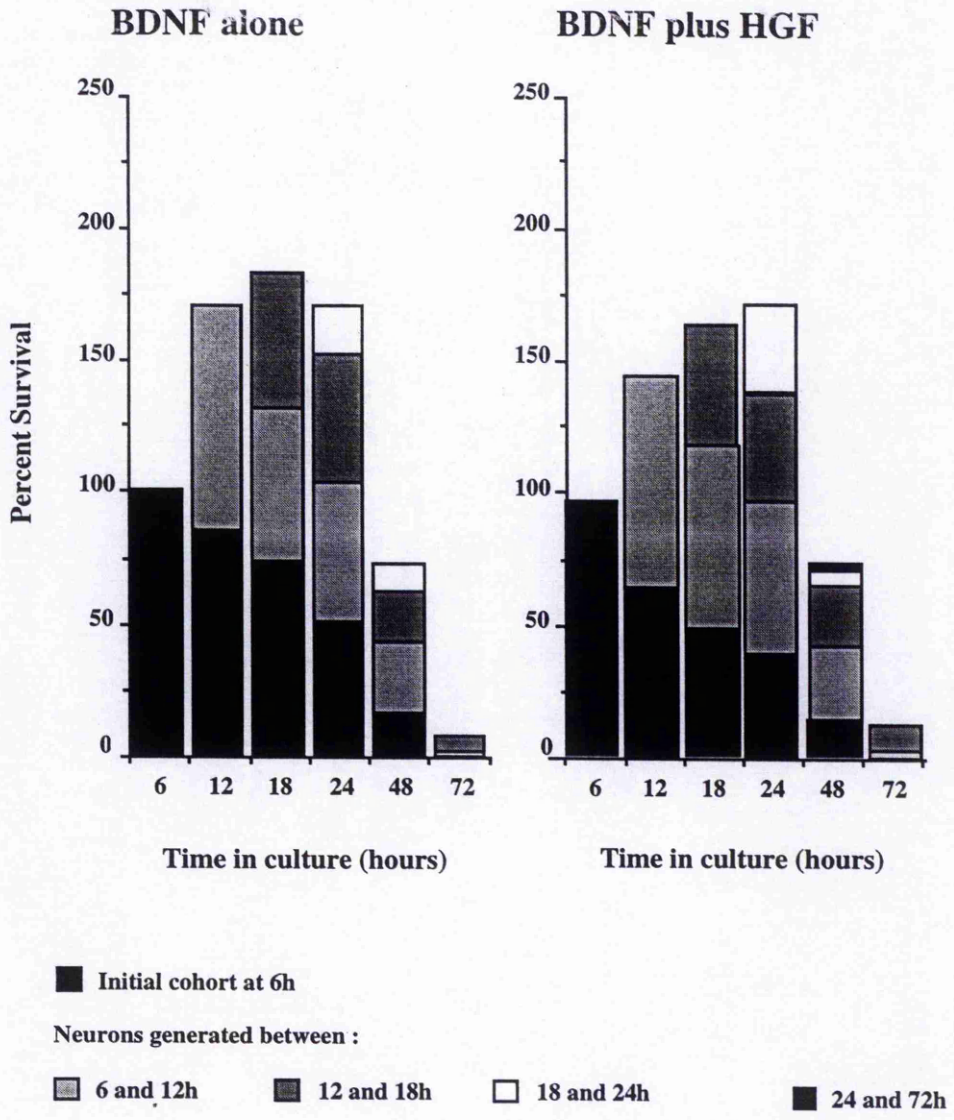


Figure 3.3: Bar charts showing the results of cohort experiments to determine the survival of thoracic DRG neurons that differentiate after different periods in culture. Neurons were grown either with 5 ng/ml of BDNF or BDNF plus 5ng/ml HGF. Similar results were obtained in three separate experiments.

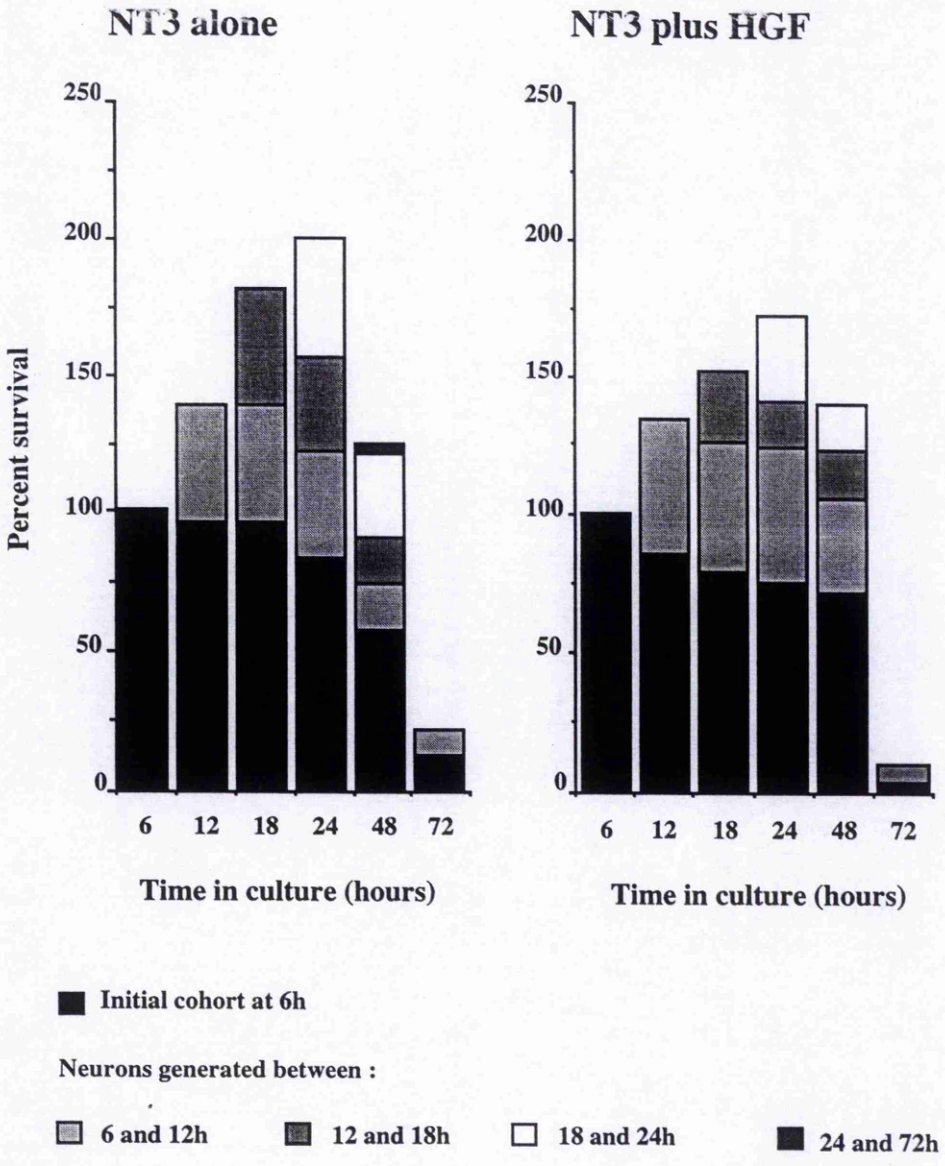


Figure 3.4: Bar charts showing the results of cohort experiments to determine the survival of thoracic DRG neurons that differentiate after different periods in culture. Neurons were grown either with 5 ng/ml of NT3 or NT3 plus 5ng/ml HGF. Similar results were obtained in three separate experiments.

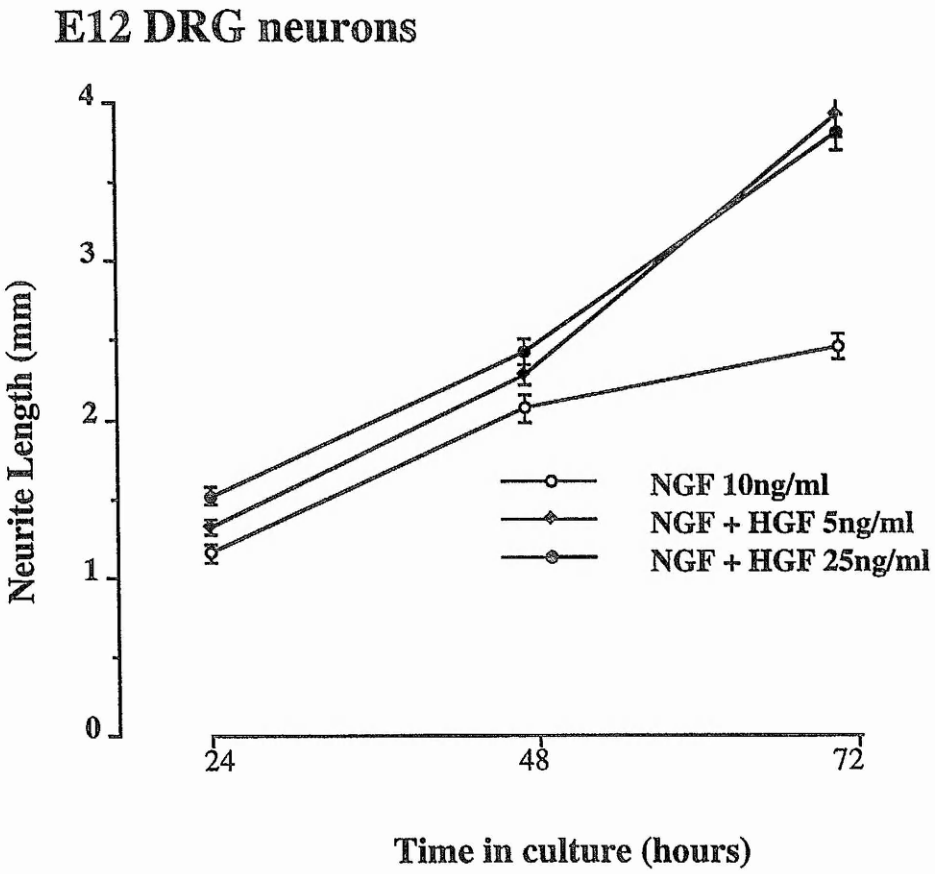


Figure 3.5: Graph of the axonal growth rates of E12 DRG neurons incubated with NGF alone or NGF plus HGF at 5ng/ml and 25ng/ml. The means and standard errors of serial measurements of between 79 and 89 neurons in each experimental group (compiled from three separate experiments) are shown.

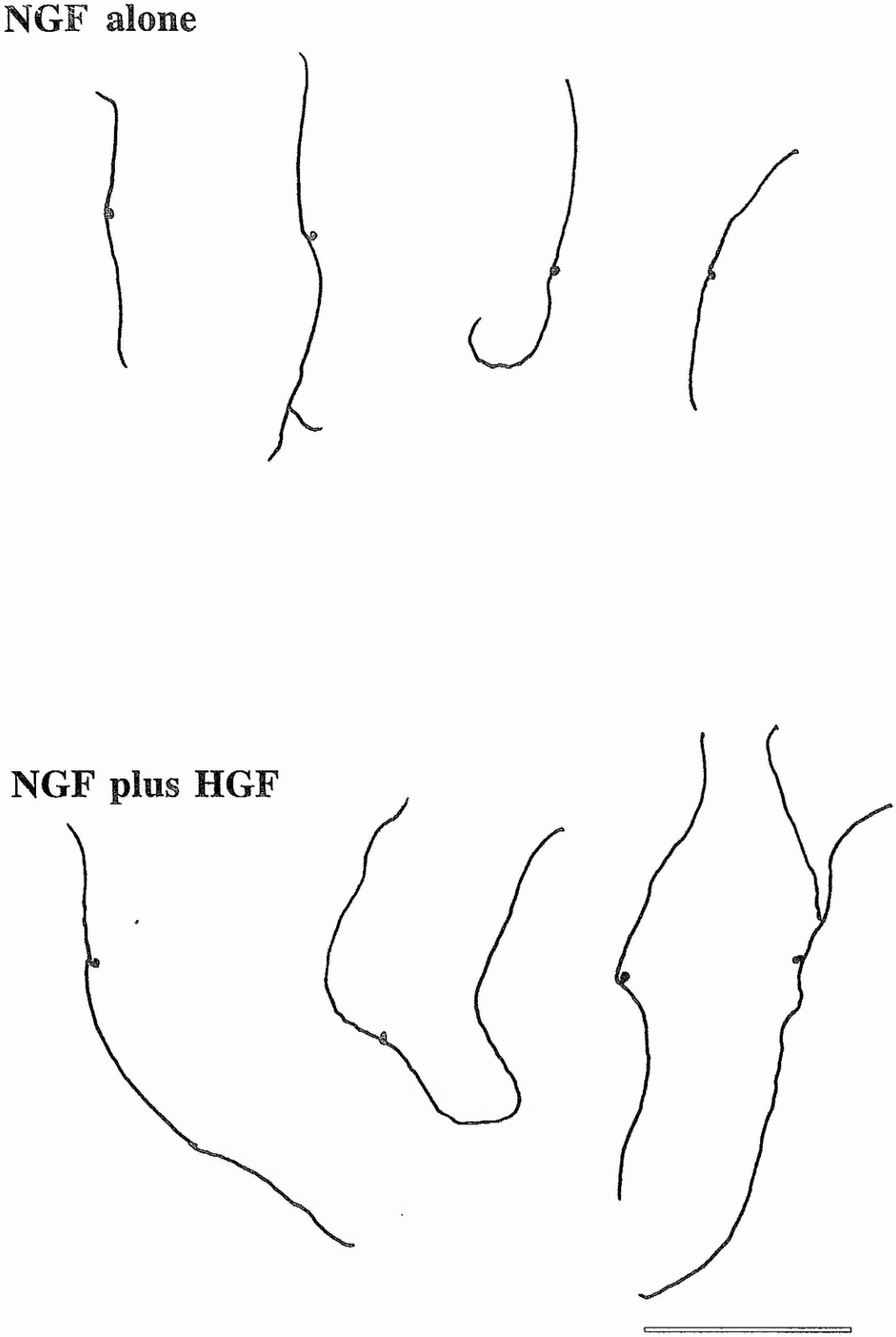


Figure 3.6: Camera lucida drawings of the typical appearance of thoracic E12 DRG neurons after being cultured in NGF and NGF plus HGF for 72 hrs. (Scale Bar = 2mm).

3.3 Discussion.

To investigate the effects of HGF on sensory neuron survival, I cultured embryonic mouse DRG neurons at low density, either without factors, with HGF alone or with HGF plus neurotrophins. DRG neurons cultured without added factors or with HGF alone died rapidly. However, there was significantly greater neuronal survival in cultures containing HGF plus NGF compared to NGF alone. Cohort studies, in which the fates of individual cells were followed, indicated that this increase in the number of surviving neurons is the result of two effects of HGF. In the presence of NGF, HGF appeared to increase the survival of differentiated neurons and to increase the number of neurons that differentiated from progenitor cells. In contrast to the enhanced survival of NGF-dependent neurons, HGF did not increase the number of sensory neurons treated with either NT-3 or BDNF. These in vitro assays show that HGF in combination with NGF but not BDNF or NT-3 can promote the differentiation and survival of DRG neurons, but cannot mediate any of these effects on its own. Measurements of axonal growth rate additionally revealed that in combination with NGF, HGF increases the growth rate of DRG axons.

The above findings suggest that within DRG neurons, the down stream signalling pathways of Met and the NGF receptor, TrKA, converge. This is different from the situation in motoneurons, where HGF alone is effective in promoting the survival of motoneurons on its own in culture (Ebens et al 1996; Wong et al 1997; Yamamoto et al 1997). Interestingly, HGF is unable to enhance the activities of NGF on another population of sensory neurons located in the trigeminal ganglion, indicating that the effects of HGF on DRG neurons are not due to a generalized increased fitness of sensory neurons (A. Forgie and A.M. Davies, unpublished observation). The results presented here raise the possibility of cell type-specific effector(s) of TrkA whose signalling is enhanced by activated Met receptors, either by stabilizing signalling activities or by blocking a specific inhibitor of the TrkA pathway. Since HGF is unable to promote survival or neurite

outgrowth in combination with NT-3 or BDNF, there is no evidence for the possible interactions between Met and TrkB or TrkC, the receptors for BDNF and NT3, respectively (Klein, 1994; Barbacid, 1995).

Analysis of the development of sensory neurons in Met mutant mice has strengthened the observations reported in this chapter and demonstrated a role for HGF/Met signalling in the development of sensory neurons *in vivo* (Maina et al., 1997). Detailed examination of the innervation of the skin of the limbs and thorax of embryos that possess a non-functional met receptor (*met*$^{d/d}$ mutant embryos) has shown that sensory nerves that are much reduced in length and elaborate fewer terminal branches. The overall patterning of the spinal nerve network appears to be preserved in *met*$^{d/d}$ mutant embryos, suggesting a role for Met signalling in terminal nerve growth and branching, rather than in initial axonal guidance. In addition to reduced growth of sensory nerve fibres in *met*$^{d/d}$ mutant embryos, there was also a consistent increase in cell death in these embryos (Maina et al., 1997). TUNEL staining of DRG sections either preceeding (E11.5) or during the period of Met-dependent neurite outgrowth (E12.5) showed a two-fold increase in the number of apoptotic cells within Dorsal root ganglia of *met* $^{d/d}$ embryos compared to littermate wild-type embryos. These results demonstrate that Met signalling is required for the survival of a subset of DRG neurons *in vivo.*

NGF and its receptor TrkA are important for the survival of pain and temperature-sensitive DRG neurons whose peripheral axons largely terminate in the skin (Crowley et al., 1994; Smeyne et al., 1994; Wall, 1995; Indo et al., 1996). The dual requirement observed *in vitro* for HGF and NGF explains the lack of superficial nerve branches in the skin of *met* $^{d/d}$ mutant embryos. NGF is currently being developed as a therapeutic drug for the treatment of peripheral sensory neuropathies, often observed in human patients suffering from diabetes or undergoing cancer therapy (Barinaga, 1994). Considering the strong enhancement of NGF-induced sprouting by HGF, combination of the two factors may prove to be even more effective (Nishi, 1994).

Chapter 4
Effects of HGF on sympathetic neurite outgrowth.

4.1 Introduction

Having demonstrated that HGF cooperates with NGF to enhance the differentiation, survival and growth of DRG neurons *in vitro* (Chapter 3) together with in vivo data that show that mouse embryos carrying a signalling mutant of Met (*met* $^{d/d}$) have fewer DRG neurons than wild type neurons and have much shorter less branched sensory nerves *in vivo* (Maina et al., 1997), I wished to extend the analysis of the function of HGF in neural development to sympathetic neurons. I was particularly interested in studying these neurons because the results of a semiquantitative RT/PCR screen of several tissues from mouse embryos mice revealed the presence of HGF and Met mRNAs in the superior cervical sympathetic ganglion (SCG) throughout its embryonic development (S. Wyatt, personal communication).

Neuroblast proliferation, differentiation and survival during the early stages of neuronal development are easily studied in paravertebral sympathetic ganglia. These ganglia can be dissected from mouse embryos as early as E12.5 when the ganglia consist predominantly of proliferating cells. These dividing cells exhibit a variety of neuronal characteristics, including; the expression of catecholamines (Cohen, 1974), dense-core vesicles, high-affinity noradrenaline uptake (Rothman et al., 1978), tyrosine hydroxylase expression (Rothman et al., 1980), tetanus toxin receptors (Rohrer and Thoenen, 1987), expression of the neuron-specific antigens SCG10, B2 (Anderson & Axel, 1986) and Q211 (Rohrer & Thoenen, 1987), neurofilament protein (DiCicco-Bloom & Black, 1988) and short neuritic processes (DiCicco-Bloom et al., 1990). These proliferating, neuron-like cells have been termed precursor cells (Anderson and Axel, 1986), neuroblasts (DiCicco-Bloom

and Black, 1988) and immature neurons (Ernsberger et al., 1989a). To distinguish these proliferating cells from post-mitotic neurons, I have used the term neuroblast.

Sympathetic neuroblasts and postmitotic sympathetic neurons initially survive in culture in the absence of added neurotrophic factors. With increasing embryonic age, an increasing proportion of sympathetic neurons become dependent on NGF for survival. The first neurons start responding to NGF at E14 and almost all are dependent on NGF by E18 (Wyatt & Davies, 1995). Between E16 and E18, the survival of some sympathetic neurons additionally becomes dependent on a supply of NT-3 in vivo (Wyatt et al., 1997), and the proportion of NT-3 dependent neurons increases in the postnatal period, during which most neurons become dependent on both or either NT-3 and NGF (Levi-Montalcini, 1987; Crowley et al., 1994; Ernfors et al., 1994; Smeyne et al., 1994; Zhou & Rush, 1995; Fagan et al., 1996; Davies, 1997; Wyatt et al., 1997; Francis et al 1999). The onset of NGF responsiveness is correlated with a marked increase in the expression of the NGF receptor tyrosine kinase, TrkA (Wyatt & Davies, 1995), which also mediates the response of the neurons to NT-3 (Davies et al., 1995; Wyatt et al., 1997).

In the studies reported in this chapter, I have addressed the question of whether HGF/Met signalling influences the differentiation, survival and axonal growth rate of neurites emanating from sympathetic neurons during embryonic development. The approach has been to follow individual neurons, calculating cell survival, neurite outgrowth and branching at regular intervals. This approach has enabled me to show that HGF/Met signalling accelerates the differentiation of sympathetic neurons as well as promoting the survival of sympathetic neuroblasts but not postmitotic neurons. HGF was also shown to cooperate with NGF to enhance the axonal growth rate as well as increasing the amount of neurite branching of NGF-dependent sympathetic neurons throughout development. To investigate the significance of Met signalling in the development of neurons, I compared the in vitro development of neurons obtained from wild-type embryos with those from embryos containing a loss of function mutation of the met receptor.

In summary these results indicatet that HGF plays several distinct roles in sympathetic neuron development at different embryonic stages.

4.2 Results

4.2.1 HGF/Met signalling accelerates the differentiation of sympathetic neurons

Sympathetic neuroblasts are proliferating neuron-like cells that make up the majority of cells in early sympathetic ganglia (Rothman et al., 1987, 1980; Rohrer and Thoenen, 1987). They have distinctive morphologies in culture with respect to post-mitotic sympathetic neurons. Whereas sympathetic neuroblasts possess small, phase-dark often irregularly shaped cell bodies and short neurites, post-mitotic sympathetic neurons have larger, spherical, phase-bright cell bodies and long neurites (Fig. 4.1). The fate of each cell type was followed over time in cohort experiments. Cells with the neuroblast morphology were often observed to divide whereas cells with the neuron morphology were never observed to divide. Because neuroblasts exhibit a variety of neuron-like characteristics (Anderson and Axel, 1986; Cohen, 1974; DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990; Rohrer and Thoenen, 1987; Rothman et al., 1978; Rothman et al., 1980), it is not possible to use a neuron-specific marker to distinguish neurons from proliferating neuroblasts in cells of developing sympathetic ganglia. Over 90% of the cells in dissociated cultures of early sympathetic ganglia (E12.5 and E13.5) of mouse embryos were neurofilament-positive neuroblasts or neurons. Most of the remaining cells had a fibroblast-like morphology.

To investigate if HGF affects the differentiation of sympathetic neuroblasts, cohorts of neuroblasts were identified in low-density dissociated cultures of E12.5, E13.5 and E14.5 SCG 6 hours after plating and the proportion of these neuroblasts that differentiated into neurons was monitored at regular intervals thereafter. As shown in Figure 4.2, anti-HGF antibodies caused a marked, significant decrease in the number of neuroblasts that differentiated into neurons compared with control cultures and cultures that were supplemented with HGF. This decrease was evident as early as 12 hours after plating, and remained significantly lower at 24 and 48

hours (*P* values ranging between 0.05 and 0.0005 at each time point; t-tests). The number of neuroblasts that differentiated into neurons was not significantly increased by HGF compared with control cultures ($p>0.05$;t-test) (Fig. 4.2). Similar effects of anti-HGF on suppressing neuroblast differentiation were observed in cultures set up from E12.5 and E14.5 SCG (Fig. 4.3), and in a limited number of experiments established from E12.5 and E13.5 thoracic paravertebral sympathetic ganglia (TPG) ($p>0.05$;t-test) (data not shown). These results suggest that endogenously produced HGF accelerates sympathetic neuroblast differentiation.

To confirm a requirement for HGF/Met signalling in the differentiation of sympathetic neuroblasts, I studied neuroblast differentiation in cohorts established from the SCG of wild-type embryos and embryos that are homozygous for a signaling mutation in the *met* gene ($met^{d/d}$), in which both phosphotyrosine residues, that act as multifunctional docking sites for SH2-containing effectors, are mutated (Maina et al., 1996). For these studies, wild-type and homozygous mutant embryos were litter mates and they derived from crossing heterozygous mice for the mutant *met* allele (i.e., $met^{d/+}$ x $met^{d/+}$). Whereas in cohorts established from the SCG of wild-type mice there were substantially fewer neurons in cultures containing anti-HGF, there were no significant differences in the number of neurons in HGF-supplemented and anti-HGF-supplemented cohorts established from $met^{d/d}$ mutant embryos (Fig. 4.4). In these experiments, all data were collected and analyzed before determining the genotypes of embryos from which the cultures were established, and were therefore uninfluenced by observer bias. These results not only confirm that HGF/Met signaling plays a role in the differentiation of sympathetic neuroblasts but show that the anti-HGF does not exert some non-specific detrimental effect on the differentiation of sympathetic neuroblasts since it does not decrease the number of neuroblasts that differentiate in cultures established from $met^{d/d}$ embryos.

4.2.2 HGF/Met signalling promotes the survival of sympathetic neuroblasts but not post-mitotic neurons

To determine if HGF affects the survival of sympathetic neuroblasts and neurons, I followed the fates of individual sympathetic neuroblasts and neurons in cohort cultures to determine how long they survived under different experimental conditions. In these experiments, cohorts of neuroblasts and neurons were identified 6 hours after plating and each cell in these cohorts was followed at regular intervals up to 72 hours in culture. Figure 4.5 plots the number of SCG sympathetic neuroblasts that die prior to differentiating into neurons. After 12 hours, neither anti-HGF nor HGF affected the number of dying cells in these cohorts. However, by 24 and 48 hours, significantly more neuroblasts had died in cultures containing anti-HGF than in control cultures or cultures supplemented with HGF (P values ranging between 0.04 and 0.0001; t-tests). There were, however, no significant differences in the number of dying neuroblasts in controls and HGF-supplemented cultures at any of these time points ($P > 0.05$; t-tests). Similar results were obtained in cohort studies of early thoracic sympathetic neuroblasts (data not shown). These results suggest that endogenously produced HGF enhances the survival of sympathetic neuroblasts. The finding that anti-HGF did not increase the number of dying neuroblasts in cultures established from *met*$^{d/d}$ embryos (data not shown) indicates that anti-HGF does not exert some non-specific toxic effect on sympathetic neuroblasts.

In contrast to sympathetic neuroblasts, HGF does not affect the survival of differentiated sympathetic neurons. Figure 4.6 shows that there was no significant difference in the number of sympathetic neurons that die in cohorts grown with NGF plus anti-HGF compared with those grown with NGF plus HGF at all time points from 12 to 72 hours in culture ($P > 0.05$; t-tests). Although sympathetic neurons die more rapidly in the absence of NGF, neither anti-HGF nor HGF affected the rate at which they die (Fig. 4.7). These experiments therefore clearly

demonstrate that HGF alone or in combination with NGF does not affect the survival of sympathetic neurons.

4.2.3 Defective Met signalling in vivo increases sympathetic neuroblast apoptosis and reduces SCG size

To determine if the in vitro effects of HGF/Met signaling on sympathetic neuroblast survival are physiologically relevant, a comparative histological study of the early SCG of wild-type and *met*$^{d/d}$ embryos was carried out in collaboration with Flavio Maina. Embryos were prepared for routine histology and the total number of cells and the number of cells with pyknotic nuclei were counted in serial sections of the SCG. At E14.5, when the SCG consists mainly of sympathetic neuroblasts, the crossectional area of the SCG of *met*$^{d/d}$ embryos was smaller than that of wild-type embryos (Figs. 4.8A and 4.8B), and there was a statistically significant reduction of 40% in cell number ($P < 0.001$, t-test, n = 11, Fig. 4.8H). The difference in crossectional area between *met*$^{d/d}$ and wild-type embryos was even more pronounced at E16 (Figs. 4.8E and 4.8F). To exclude the possibility that the reduction in the size of the SCG in *met*$^{d/d}$ embryos is a non-specific secondary consequence of the placental defect in these embryos, we counted the number of neurons in the nodose ganglia (a cranial sensory ganglion that lies next to the SCG) in E14.5 wild-type and *met*$^{d/d}$ embryos. There was no statistically significant difference in the number of neurons in the nodose ganglia of *met*$^{d/d}$ and wild-type embryos ($P = 0.4$, t-test, n = 12, Fig. 4.8I).

There was a significant, 79% increase in the number of pyknotic nuclei in the SCG of *met*$^{d/d}$ embryos compared with wild-type embryos ($P < 0.005$, t-test, n = 12, Fig. 4.8G). In addition to recognising apoptotic cells by histological criteria, apoptotic cells were also labeled in a limited number of embryos by TUNEL staining. The number of TUNEL-positive cells was also clearly greater in the SCG of *met*$^{d/d}$ embryos compared with wild-type embryos, both at E13.5 (data not shown) and E14.5 (Figs. 4.8C and 4.8D). These results suggest that in the absence

of Met signaling, an increased proportion of neuroblasts undergo apoptosis in the early SCG and this is reflected in a reduction in the total number of cells in this ganglion.

Figure 4.1: Phase contrast micrograph to show two representative post-mitotic sympathetic neurons with spherical cell bodies and long neurites (top and bottom) and a dividing sympathetic neuroblast with darkly stained cell bodies and short processes. SCG dissected neurons from E14.5 embryos were cultured for 20h in the presence of NGF. (Scale bar = 1mm).

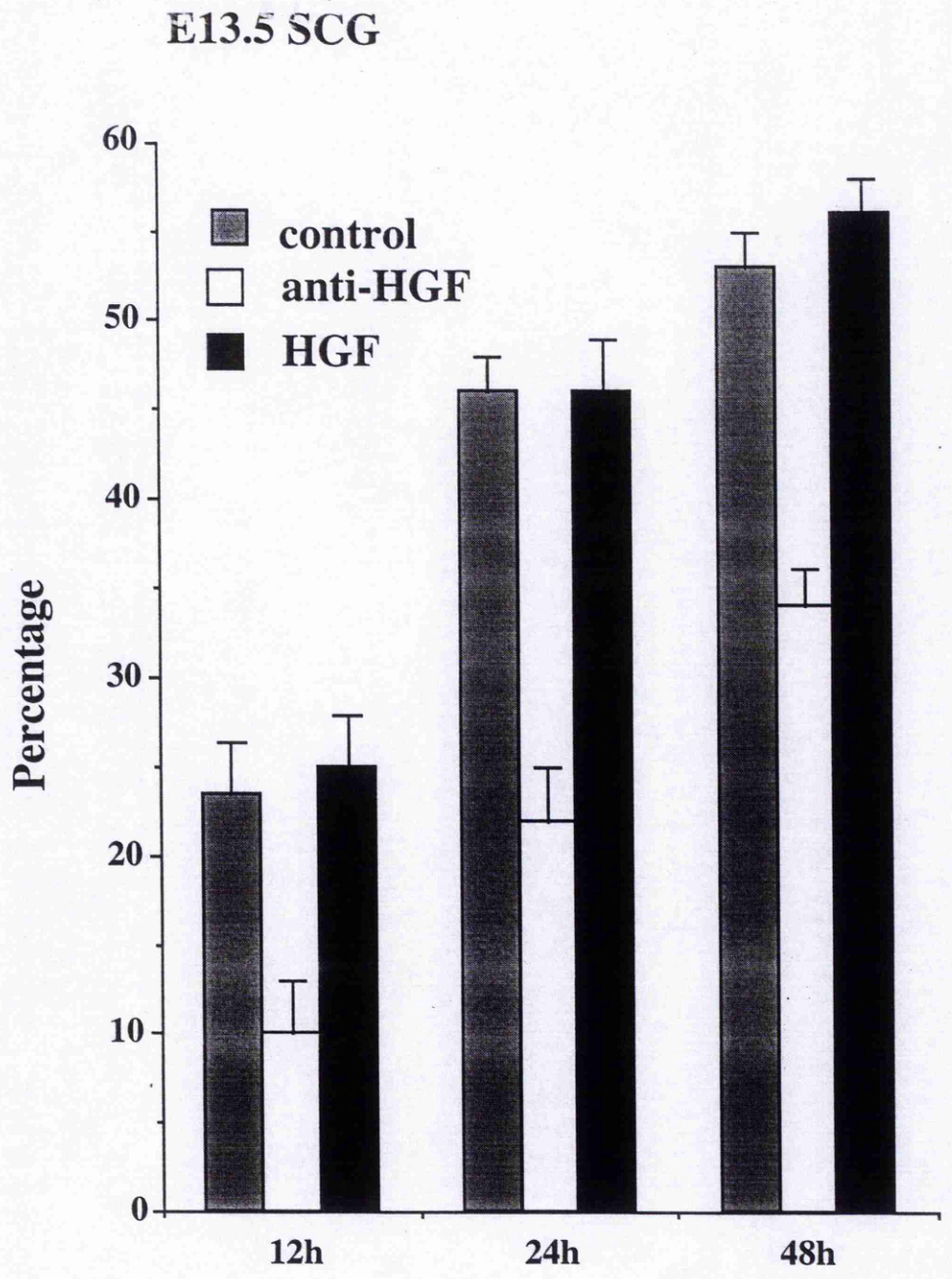


Figure 4.2: Bar graph showing the Percentage of E13.5 SCG sympathetic neuroblasts that differentiate over 48 hours in culture without factors, with anti-HGF (1.25µg/ml) or with HGF (10ng/ml). The means and standard errors of serial number of differentiated neuroblasts of between 75 and 165 neurons in each experimental group (compiled from three to four separate experiments) are shown.

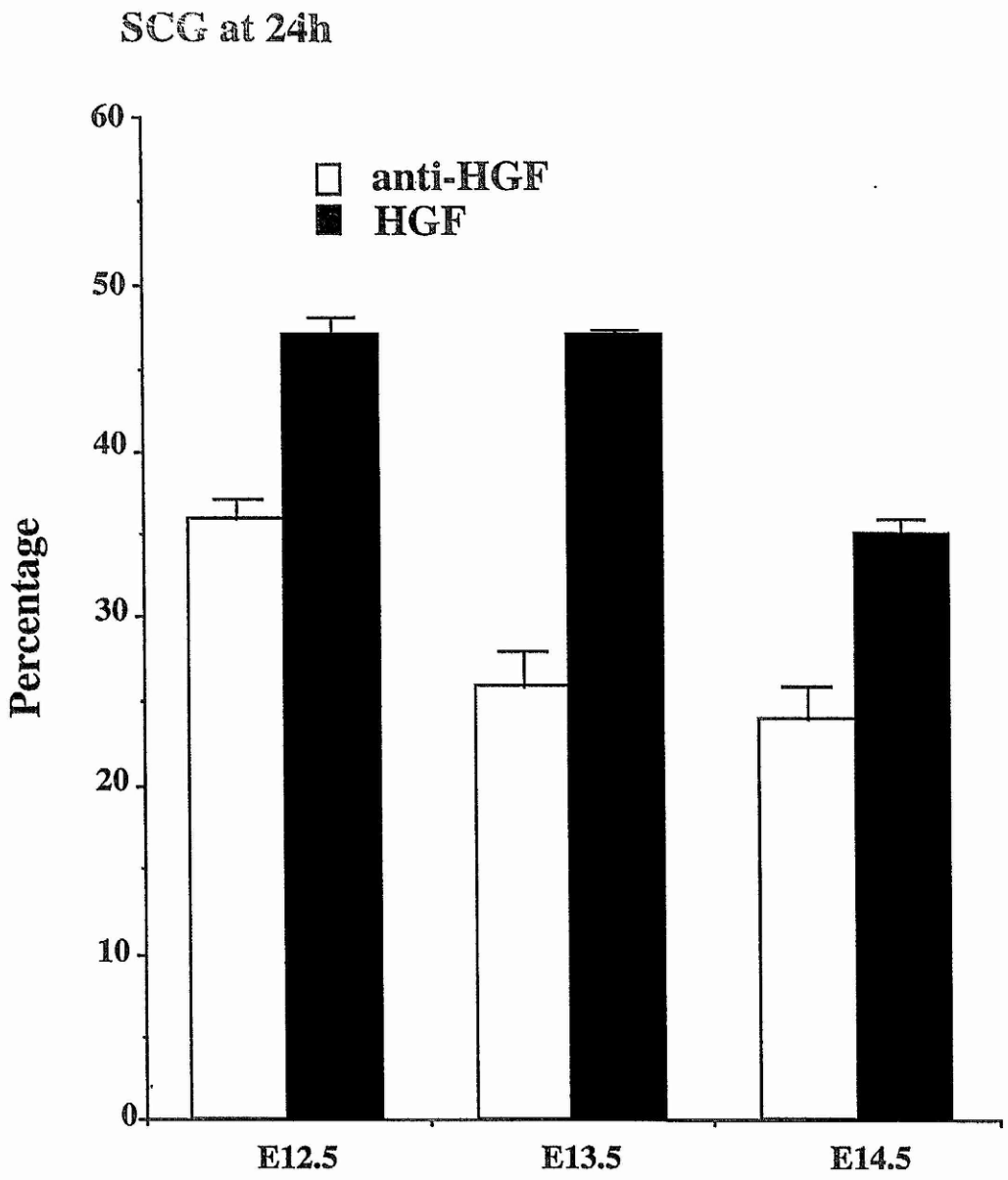


Figure 4.3: Bar graph showing the percentage of E12.5, E13.5 and E14.5 SCG sympathetic neuroblasts that differentiate after 24 hours in the presence of anti-HGF (1.25 mg/ml) or HGF (10 ng/ml). The means and standard errors of serial number of differentiated neuroblasts of between 75 and 165 neurons in each experimental group (compiled from three to four separate experiments) are shown.

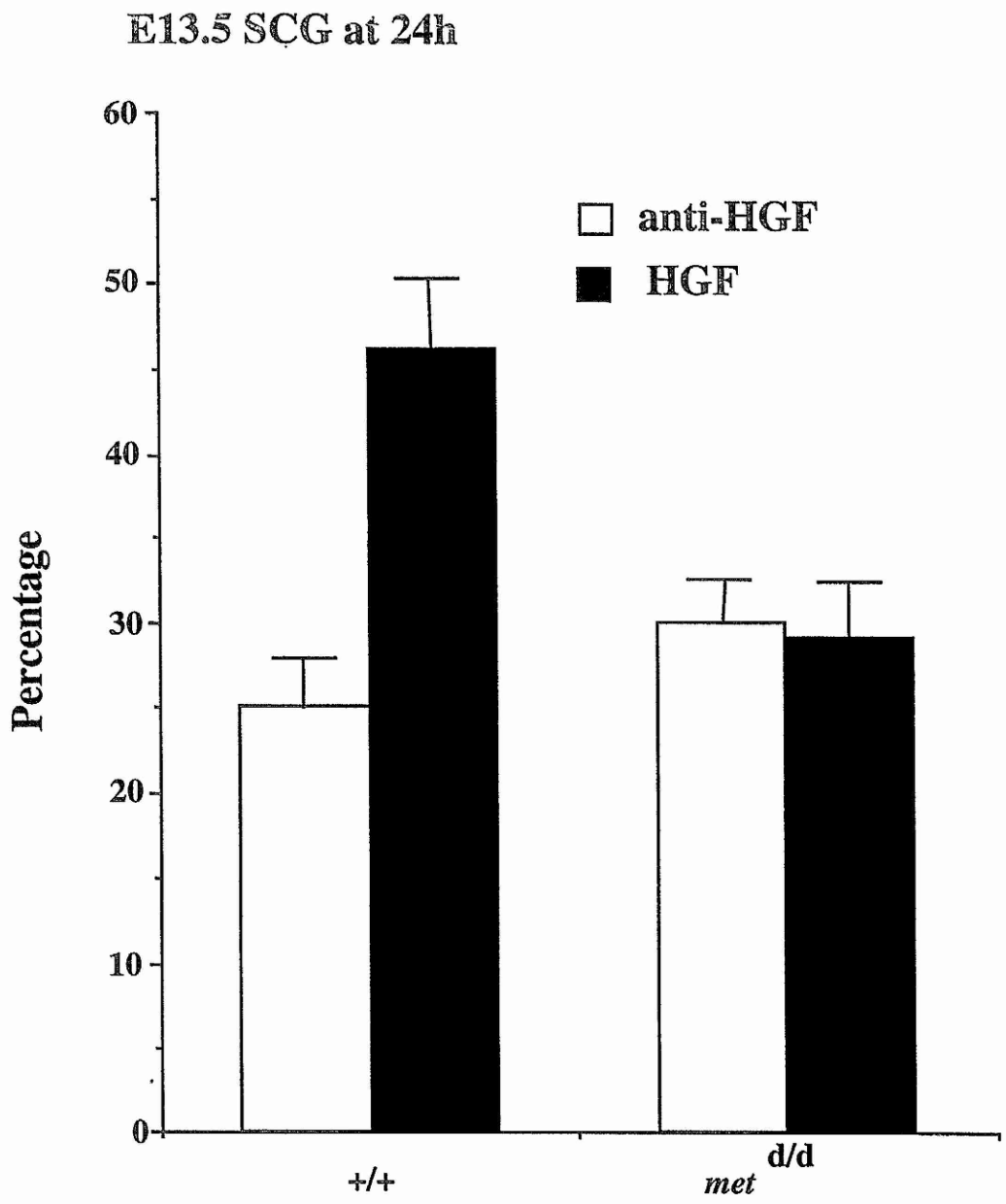


Figure 4.4: Bar graph showing the percentage of E13.5 SCG sympathetic neuroblasts from wild-type and *met*$^{d/d}$ embryos that differentiate after 24 hours in culture with anti-HGF (1.25 mg/ml) or HGF (10 ng/ml). The means and standard errors of serial number of differentiated neuroblasts of between 75 and 165 neurons in each experimental group (compiled from three to four separate experiments) are shown.

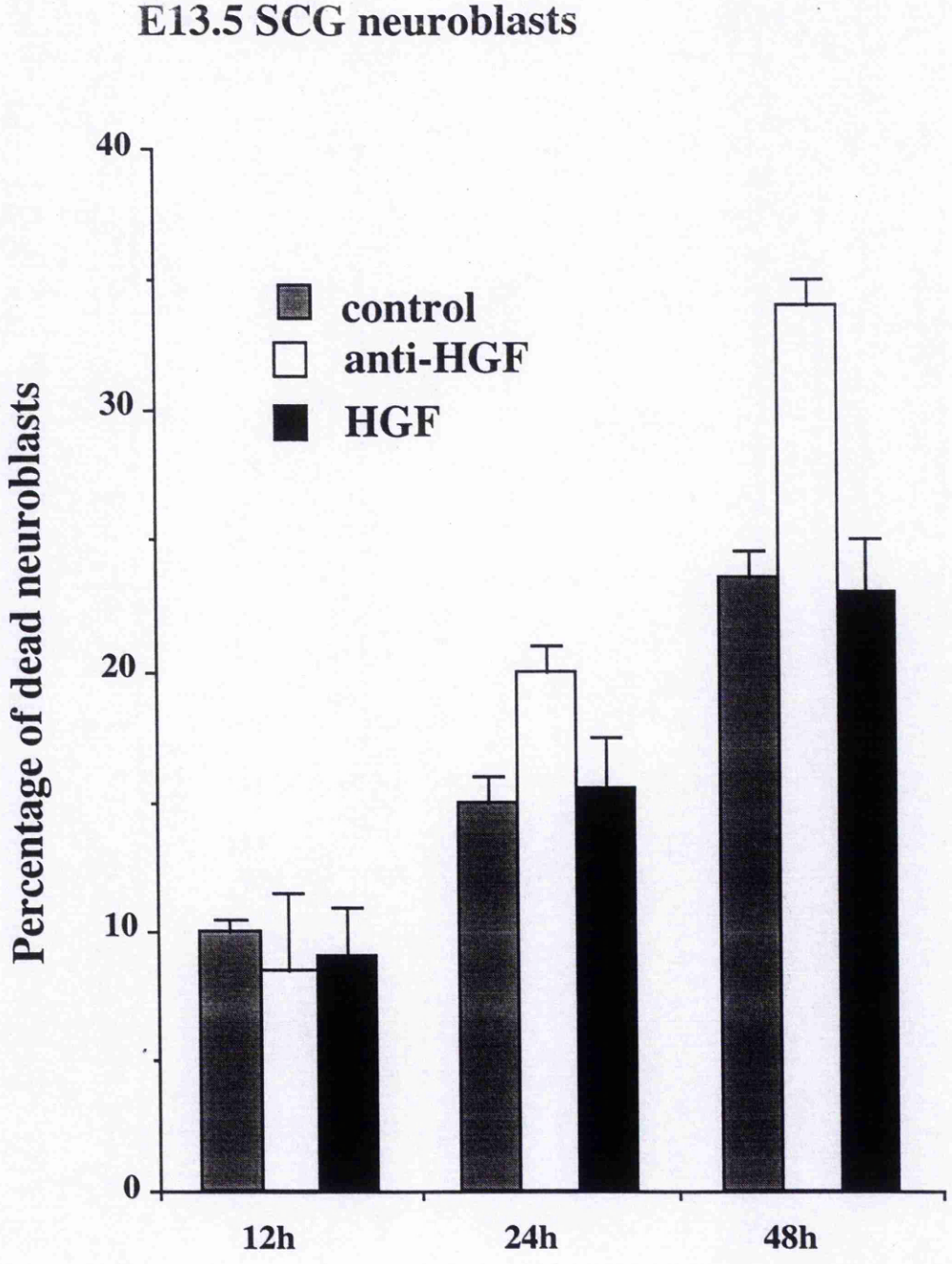
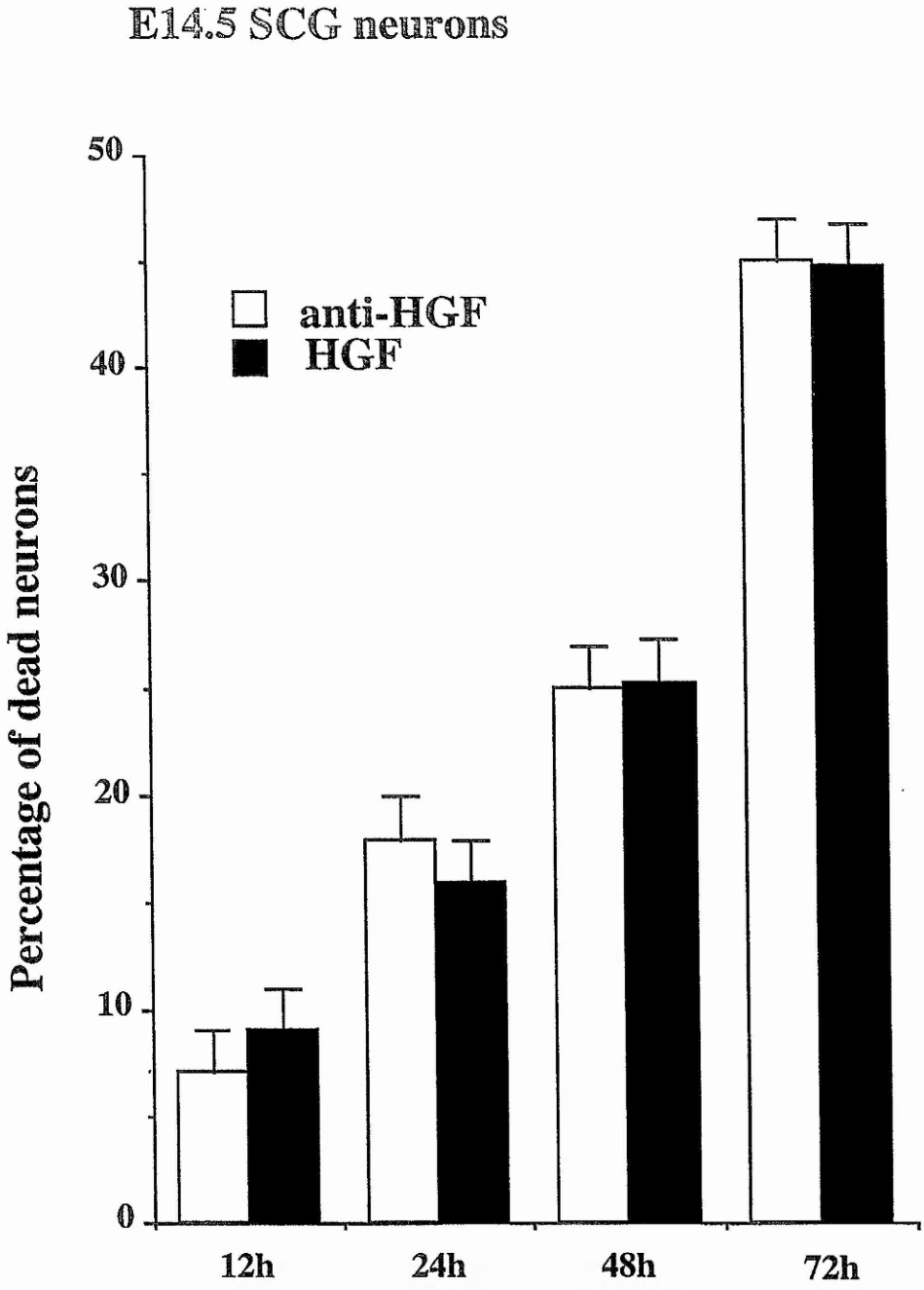


Figure 4.5: Bar graph showing the percentage of SCG sympathetic neuroblasts that die prior to differentiating into neurons over 48 hours in culture without factors, anti-HGF (1.25µg/ml), or with HGF (10ng/ml). The means and standard errors of serial number of differentiated neuroblasts of between 75 and 165 neurons in each experimental group (compiled from three to four separate experiments) are shown.



.**Figure 4.6:** Bar graph showing the percentage of post-mitotic sympathetic that die at different times in cultures supplemented with NGF (2ng/ml) plus anti-HGF (1.25μg/ml) or NGF plus HGF (10ng/ml). The means and standard errors of serial numbers of surviving neuroblasts or neurons of between 70 and 100 neurons in each experimental group (compiled from three separate experiments) are shown.

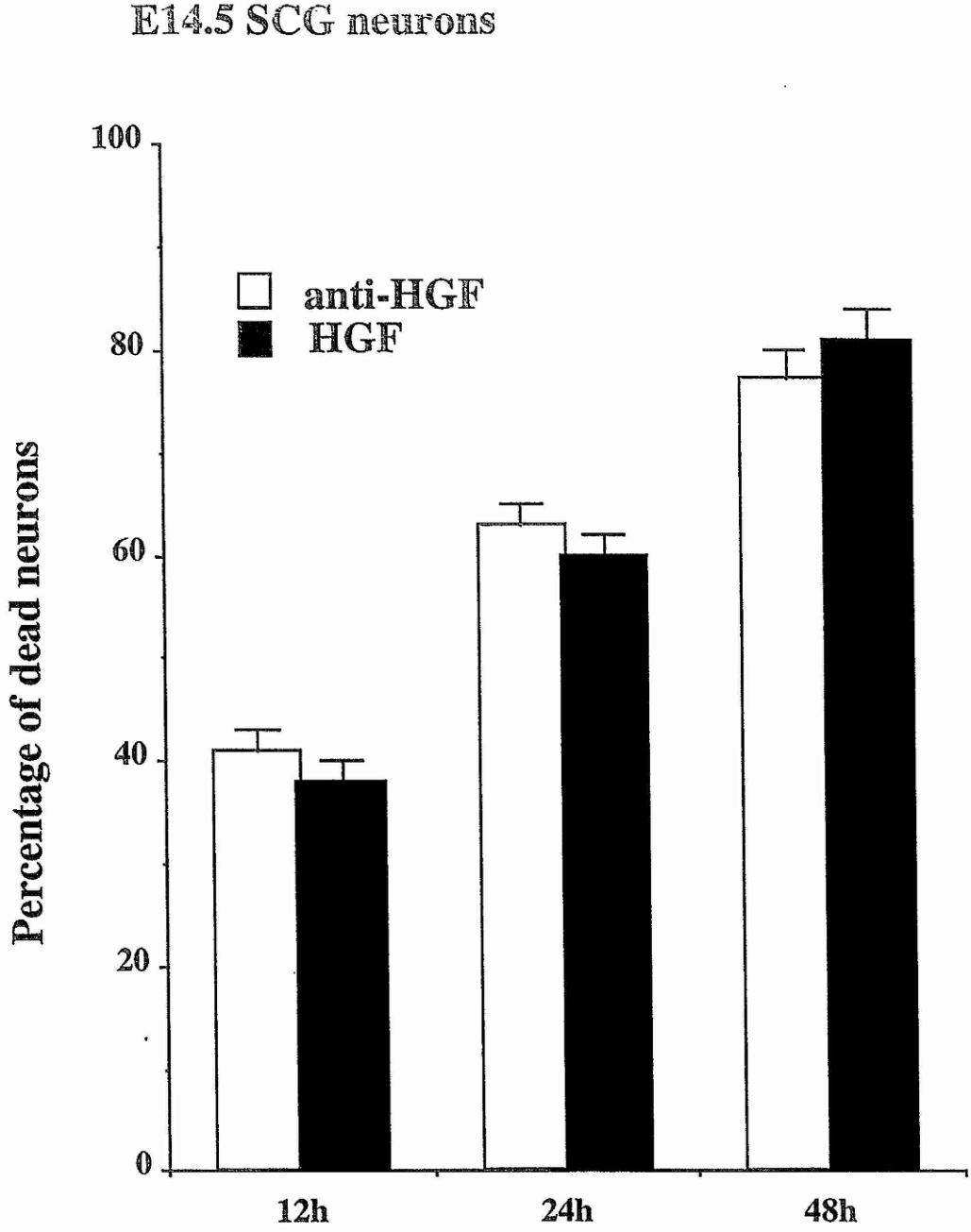


Figure 4.7: Bar graph showing the percentage of post-mitotic sympathetic that die at different times in cultures supplemented with HGF (10ng/ml) or with anti-HGF (1.25µg/ml). The means and standard errors of serial numbers of surviving neuroblasts or neurons of between 70 and 100 neurons in each experimental group (compiled from three separate experiments) are shown.

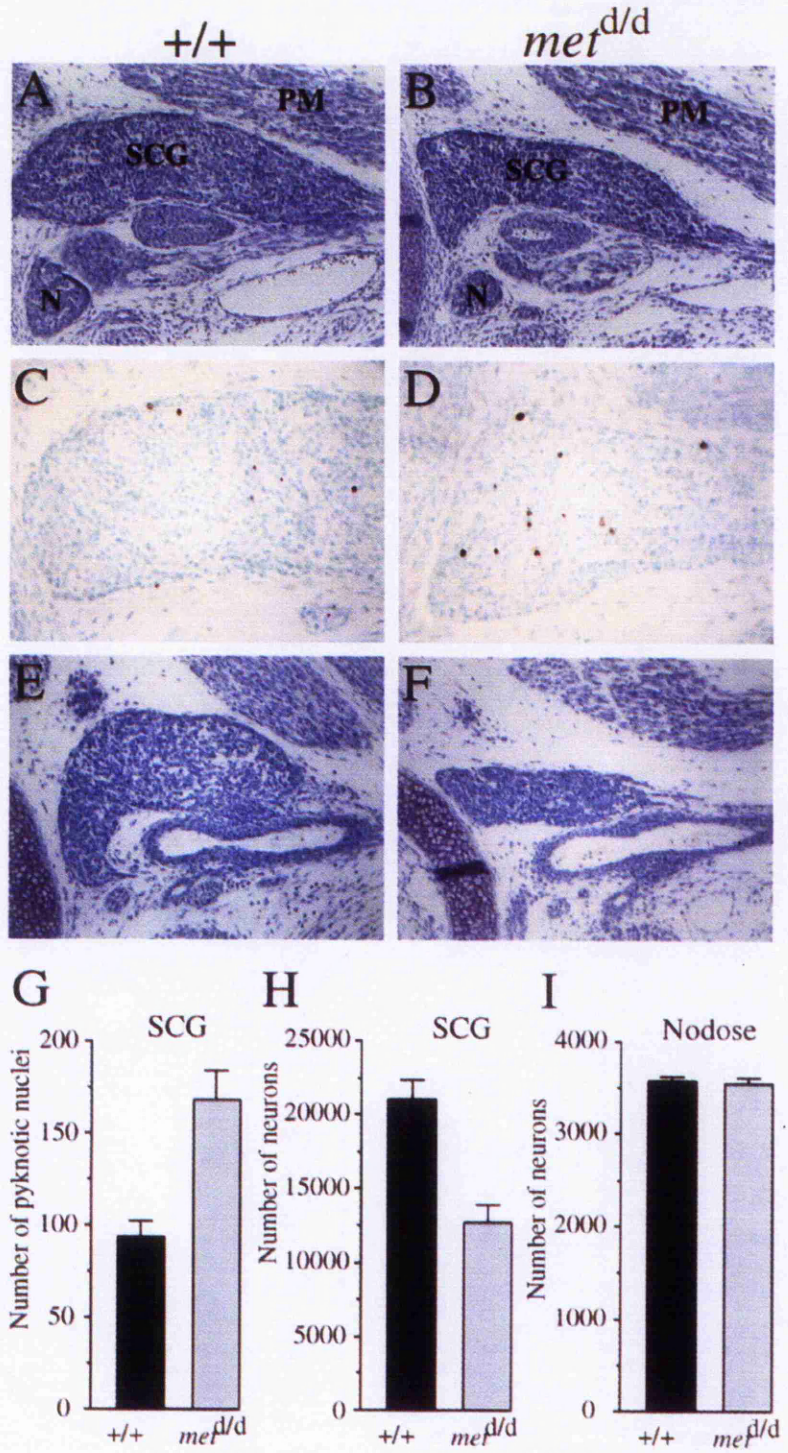


Figure 4.8: Photomicrographs of equivalent crossections of SCGs from E14.5 (A-D) and E16.0 (E,F) wild-type (+/+) (A,C,E) and *met*$^{d/d}$ mutant embryos (B,D,F) stained with cresyl violet (A,B,E,F) or by the TUNEL technique (C,D). Note the decrease in size and the increase in TUNEL-positive cells in *met*$^{d/d}$ mutant SCGs. (G,H,I) Quantitative analysis of the number of pyknotic nuclei (G) and the number of neurons in SCG (H) and nodose ganglia (I) derived from E14.5 wild-type (+/+) and *met*$^{d/d}$ mutant embryos.

4.2.4 HGF cooperates with NGF to enhance axonal Growth of Sympathetic neurites.

To investigate the effects of HGF/Met signalling on the growth of neurites emanating from sympathetic neurons, I examined the effects of HGF on neurons cultured from the SCG and TPG at different stages during embryonic development. Because HGF mRNA is expressed in developing sympathetic ganglia, I used function blocking anti-HGF antiserum to inactivate HGF produced in some cultures and made serial drawings of neurons grown with NGF alone, NGF plus anti-HGF and NGF plus HGF. The total length of each neurite arbor was measured at intervals by digitising the drawings. To avoid any confusion about which neurites emanated from each cell body, the neurons were grown at very low density so that their neurite arbors did not overlap. To avoid the effects of HGF on neuronal differentiation and survival biasing the data, serial drawings were made of the same neurons between 3 hours and 72 hours (Davies, 1989) and only neurons that survived throughout this period were included in the analysis.

After 48 hrs in culture, the mean neurite length of SCG neurons (Fig. 4.9) and TPG (Fig. 4.10) neurons cultured with HGF plus NGF, was shown to be significantly greater than neurons cultured in NGF alone or NGF plus anti-HGF, at all ages studied (E12.5, E13.5, E14.5, and E15.5; $p < 0.0005$ in all cases; t tests). The difference in neurite outgrowth was most marked in E14.5 TPG neurons whose average neurite length was observed to be 2-fold longer in the presence of HGF plus NGF compared to NGF alone or NGF plus anti-HGF. Although the neurite outgrowth from neurons cultured with anti-HGF plus NGF was generally less than that from neurons cultured with NGF alone, the differences were not significant ($p > 0.05$; t tests) in all cases except for E14.5 and E15.5 SCG neurons ($p < 0.05$; t tests). Having demonstrated an effect of HGF on neurite outgrowth, I then examined the effect of HGF on SCG (Fig. 4.11) and TPG (Fig. 4.12) neurons at 24 hour intervals over 72 hrs. After 24 hrs in culture, there was no significant difference in neurite outgrowth between SCG and TPG neurons cultured

with NGF plus HGF or NGF plus anti-HGF. However, between 24 and 48 hrs neurite outgrowth in HGF-supplemented cultures increased much more rapidly than neurons supplemented with anti-HGF resulting in significantly longer neurites in HGF-supplemented cultures at 48 and 72 hrs ($p < 0.0001$; t test).

To determine the range of concentrations over which HGF enhances sympathetic neurite outgrowth in vitro, E14.5 SCG (Fig. 4.13) and TPG (Fig. 4.14) neurons were cultured with HGF over a wide range of concentrations (3.2 pg/ml-50ng/ml), either with or without 2ng/ml NGF. In the case of both SCG and TPG neurons the average neurite length in cultures supplemented with NGF and HGF was significantly increased when HGF was present at concentrations greater than 10ng/ml ($p< 0.002$; t test). This increase was critically dependent on the presence of NGF. To determine the range of concentrations of NGF over which HGF enhances sympathetic neurite outgrowth, E14.5 TPG and SCG neurons were cultured with a constant concentration of HGF (10ng/ml) plus NGF over a wide range of concentrations (3.2 pg/ml-50ng/ml). In SCG neurons (Fig. 4.15), neurite outgrowth of HGF-supplemented neurons was significantly greater in the presence of NGF at concentrations ranging from 2-5 ng/ml ($p< 0.05$, t tests). In TPG neurons (Fig. 4.16), neurite outgrowth of HGF-supplemented neurons was significantly greater in the presence of NGF at concentrations ranging from 0.4-10 ng/ml ($p< 0.005$; t tests) but not at concentrations above or below this. These results clearly show that the neurite outgrowth of sympathetic neurons is regulated by NGF and HGF over a wide range of concentrations and combinations.

To confirm a requirement for Met signalling in mediating the effects of HGF on neurite growth, I studied the neurite outgrowth of sympathetic neurons from wild-type embryos and $met^{d/d}$ signaling mutant embryos. Whereas the neurites of SCG neurons (Fig. 4.17) and TPG neurons (Fig. 4.18) from wild-type embryos were significantly longer in medium containing NGF plus HGF than in medium containing NGF plus anti-HGF ($P < 0.0001$; t-tests), there was no significant difference in the length of neurite of SCG and TPG neurons from $met^{d/d}$ embryos

grown in medium containing NGF plus HGF compared to medium containing NGF plus anti-HGF ($P > 0.05$; t-tests). Moreover, there was no significant difference in the length of neurites of neurons from *met*$^{d/d}$ embryos compared to neurons from wild-type embryos grown with NGF plus anti-HGF ($P > 0.05$ in all cases; t-tests). These results suggest that HGF/Met signalling plays a role in enhancing the overall length of sympathetic neurite arbors and show that anti-HGF does not exert a non-specific detrimental effect on neurite growth since it does not decrease neurite length in cultures established from *met*$^{d/d}$ embryos.

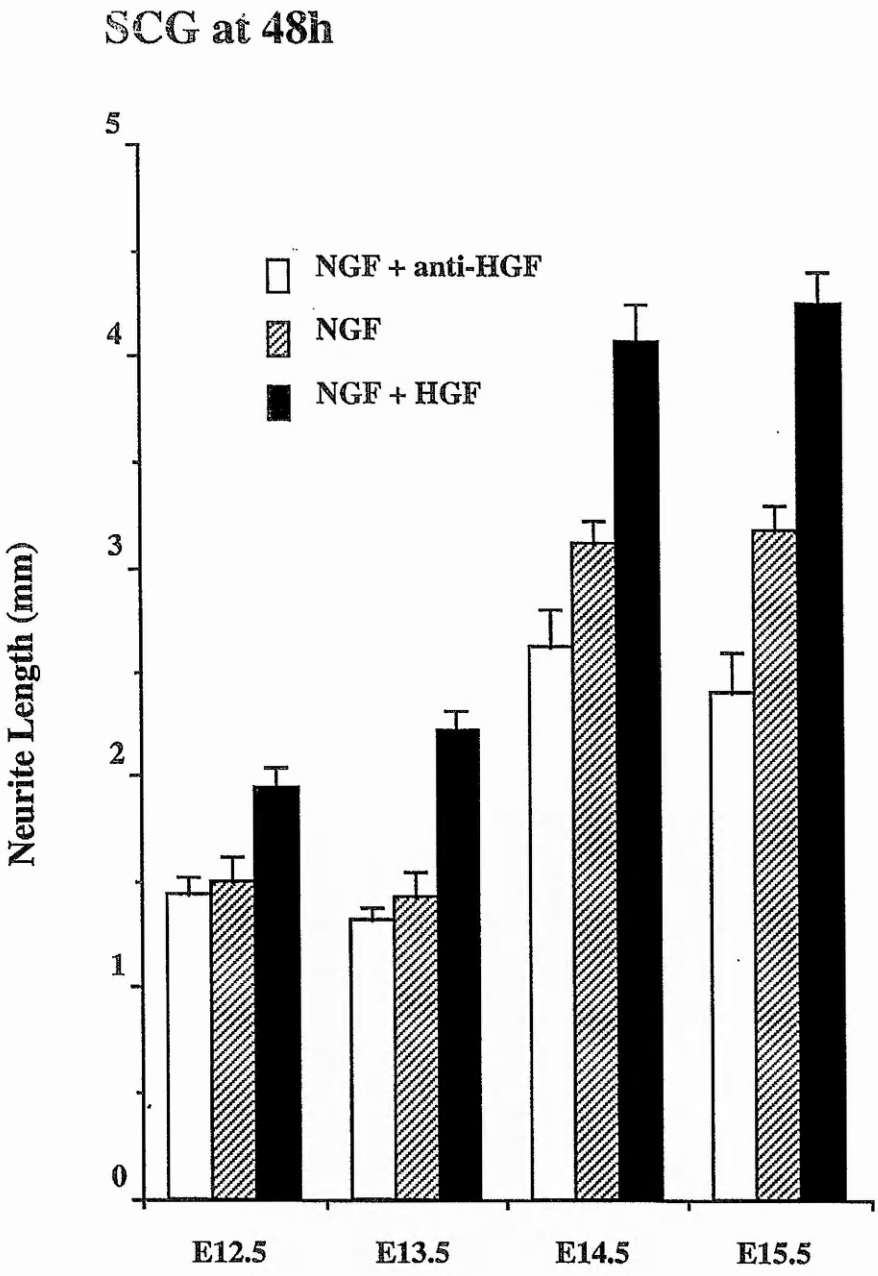


Figure 4.9: Bar graphs showing the results of neuron cohort experiments comparing neurite length of SCG neurons from different aged embryos after 48 hrs in culture with NGF (5ng/ml), NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

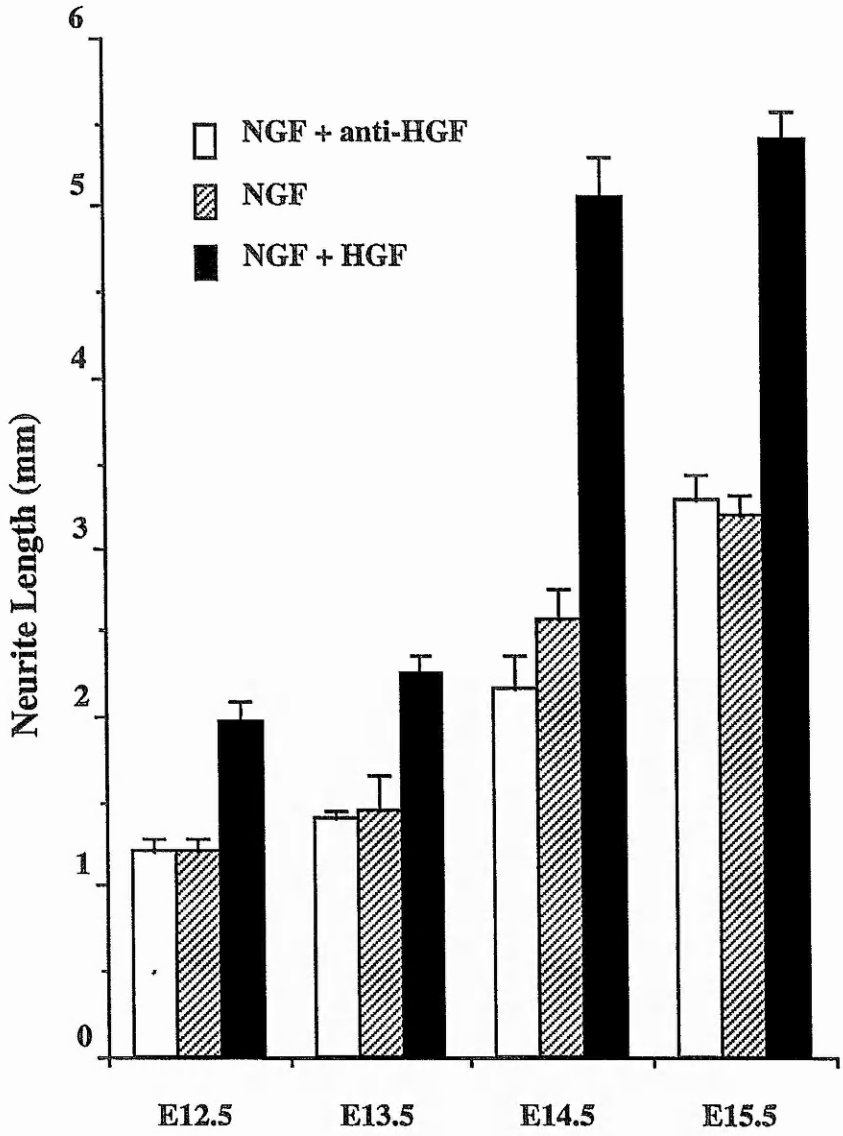


Figure 4.10: Bar graphs showing the results of neuron cohort experiments comparing neurite length of TPG neurons from different aged embryos after 48 hrs in culture with NGF (5ng/ml), NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

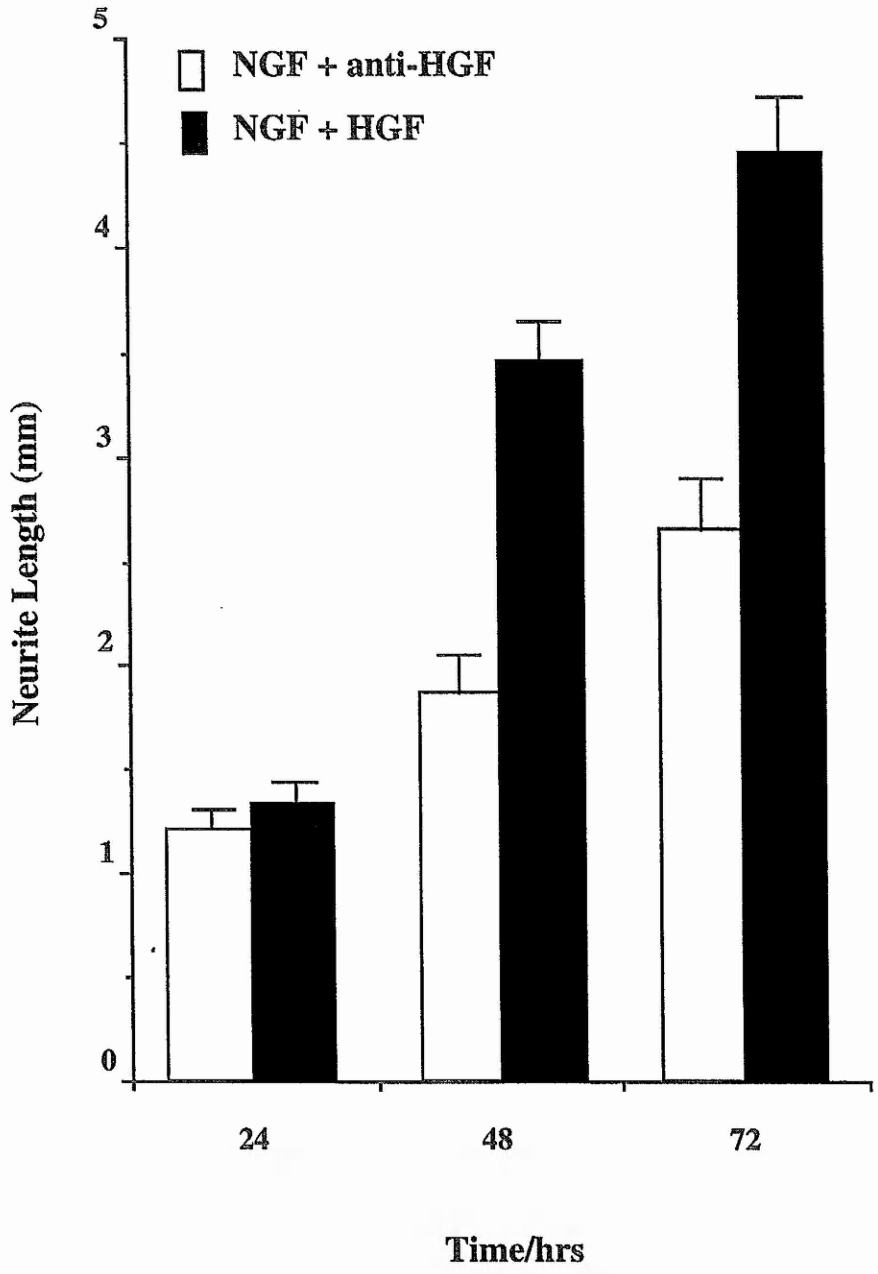


Figure 4.11: Bar graphs showing the results of neuron cohort experiments comparing neurite length of SCG neurons from E14.5 embryos cultured with NGF (5ng/ml), NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml) over 72hrs in culture. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

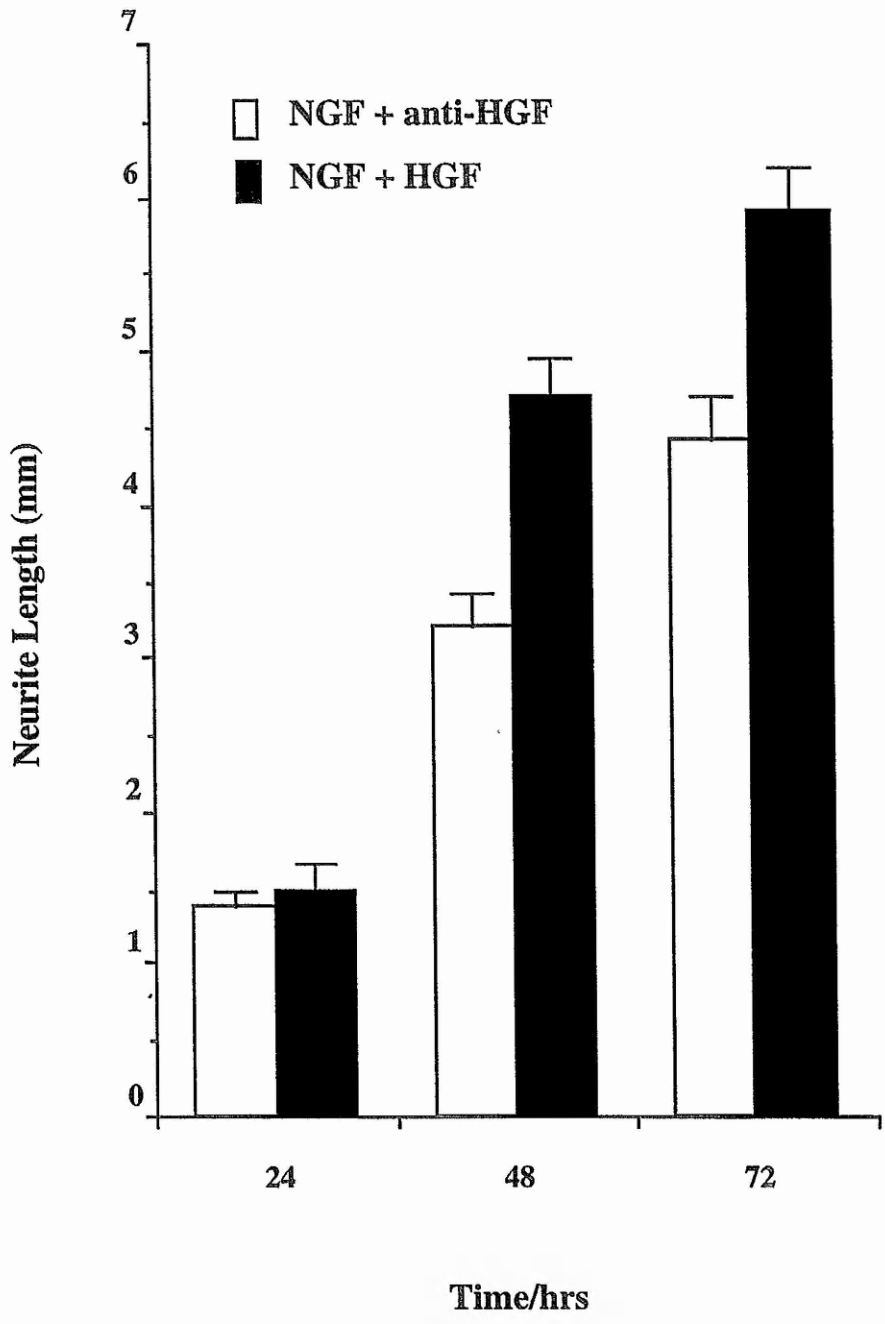


Figure 4.12: Bar graphs showing the results of neuron cohort experiment comparing neurite length of TPG neurons from E14.5 embryos cultures in NGF (5ng/ml), NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml) over 72hrs in culture. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

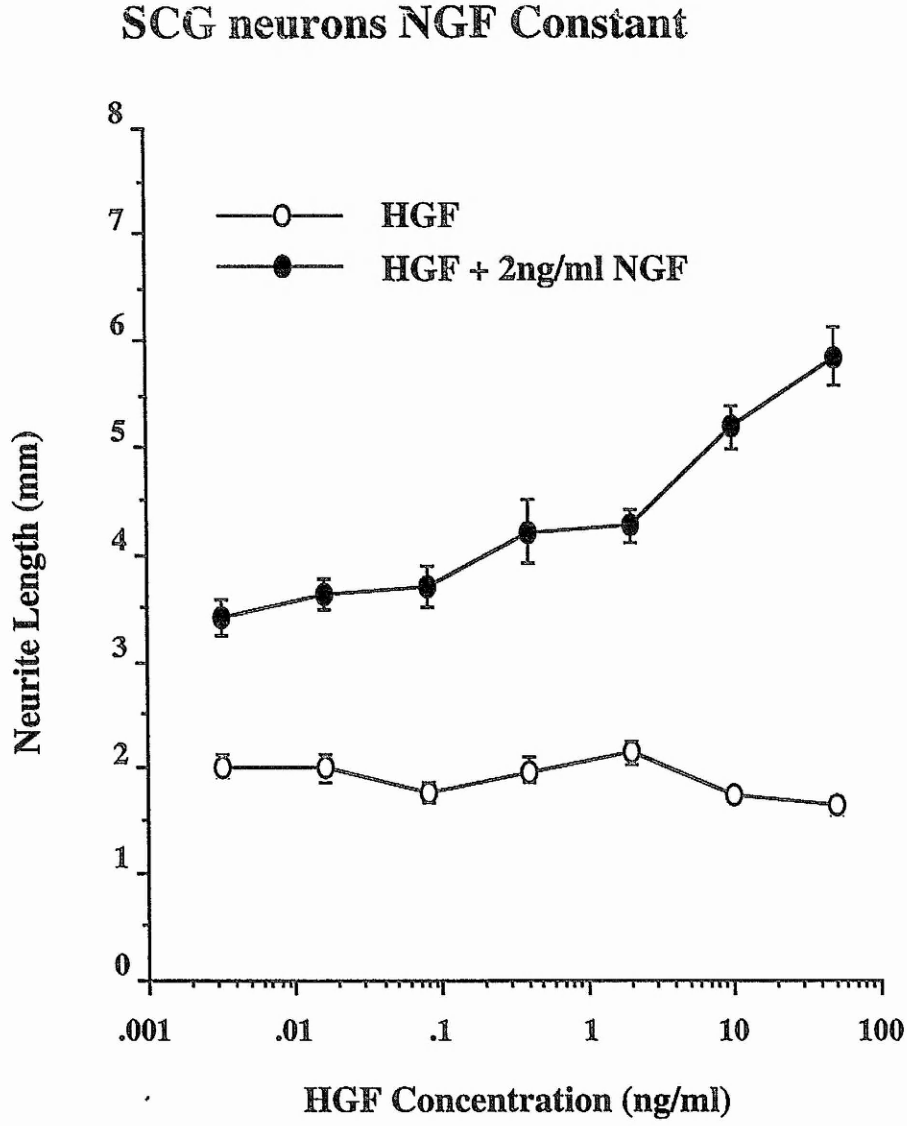


Figure 4.13: Dose response of the HGF effect on E14.5 SCG neurons. The graph shows neurite length after 48 hrs in culture. Neurons were cultured with a range of HGF concentrations either with or without 2ng/ml NGF. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

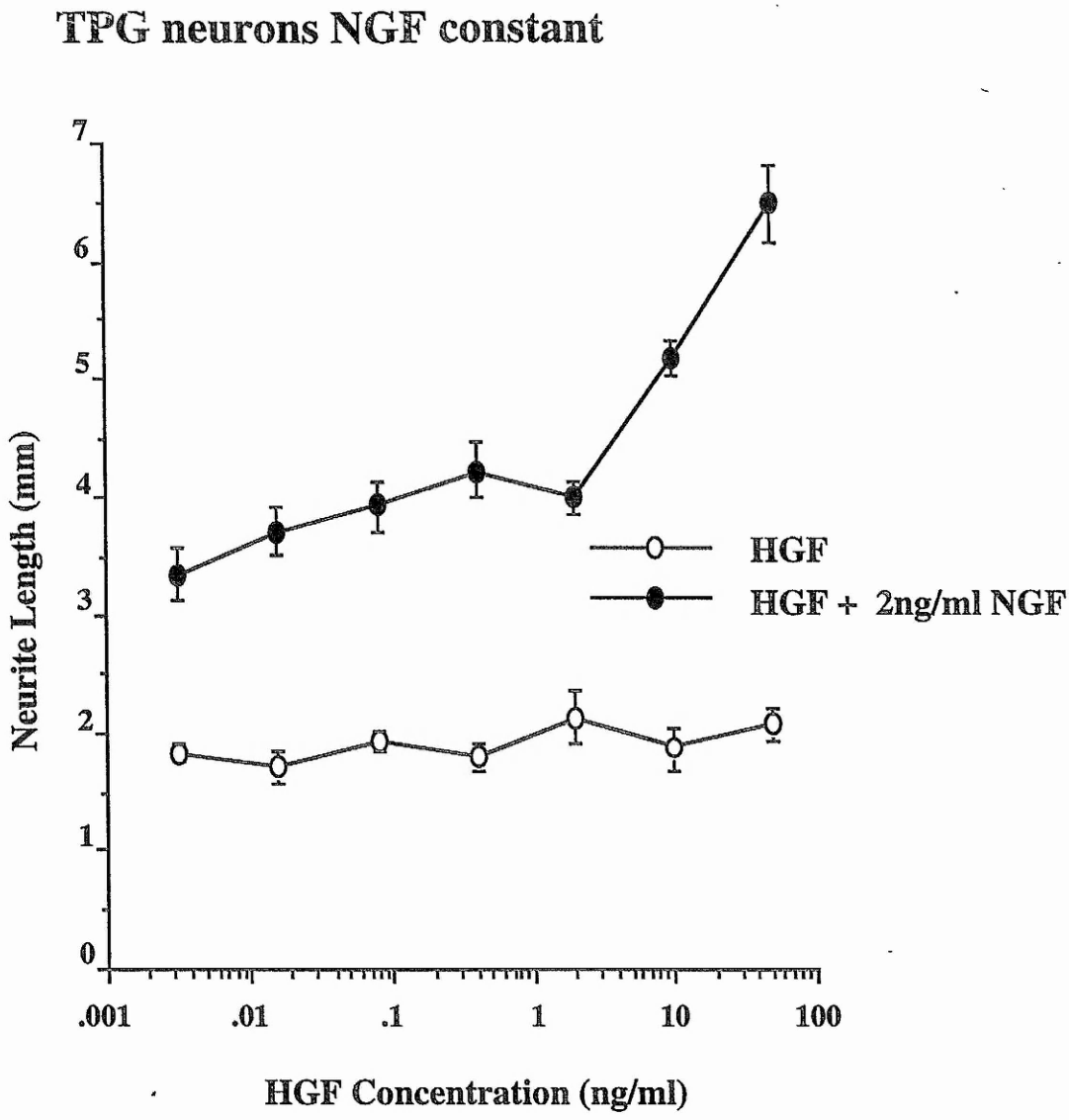


Figure 4.14: Dose response of the HGF effect on E14.5 TPG neurons. The graph shows neurite length after 48 hrs in culture. Neurons were cultured with a range of HGF concentrations either with or without 2ng/ml NGF. The means and standard errors are shown of measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

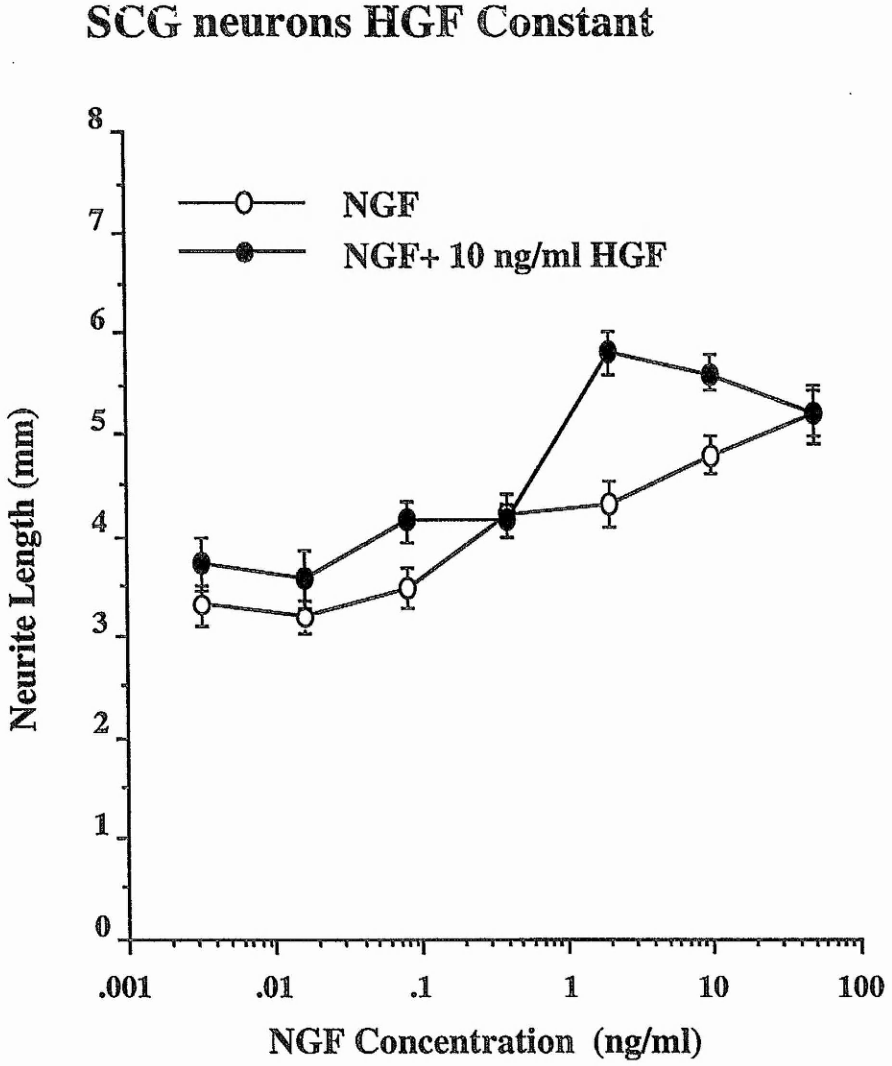


Figure 4.15: Dose response of the NGF effect on E14.5 SCG neurons. The graph it shows neurite length after 48 hrs in culture. Neurons were cultured with a range of NGF concentrations either with or without 10ng/ml HGF. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

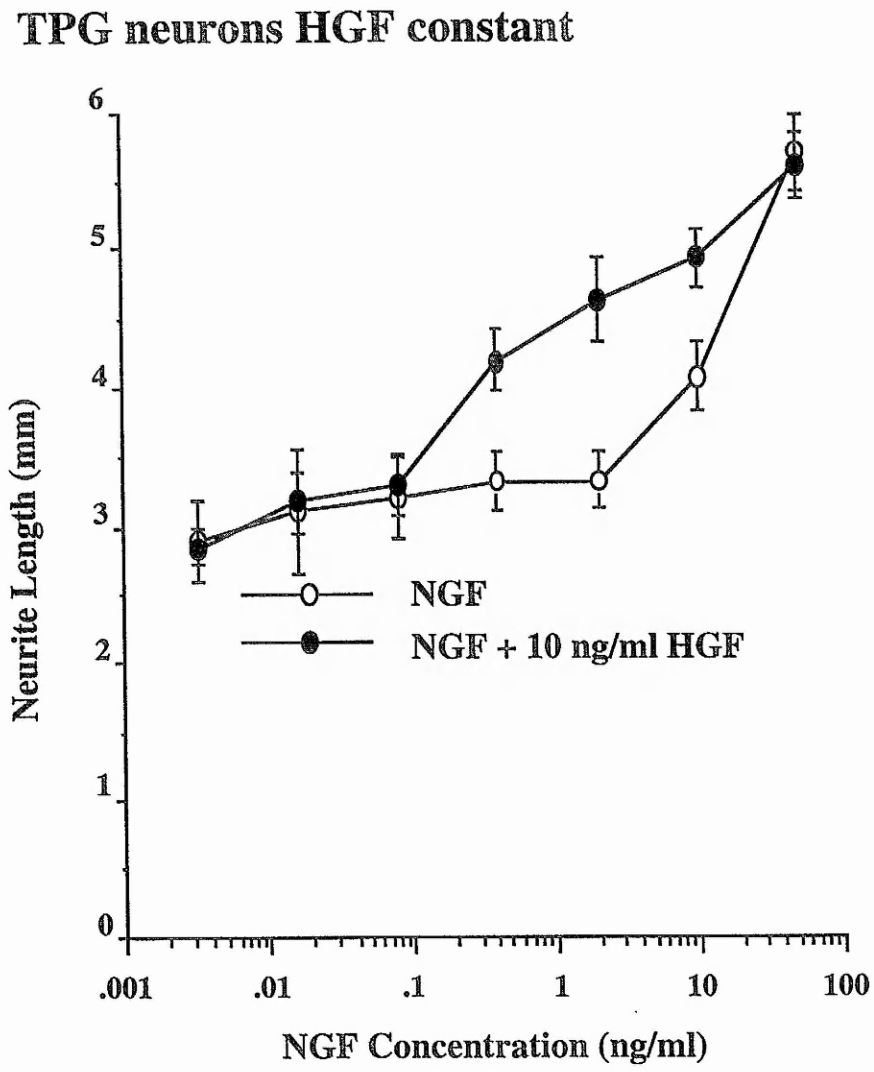


Figure 4.16: Dose response of the NGF effect on E14.5 TPG neurons. The graph shows neurite length after 48 hrs in culture. Neurons were cultured with a range of NGF concentrations either with or without 10ng/ml HGF. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

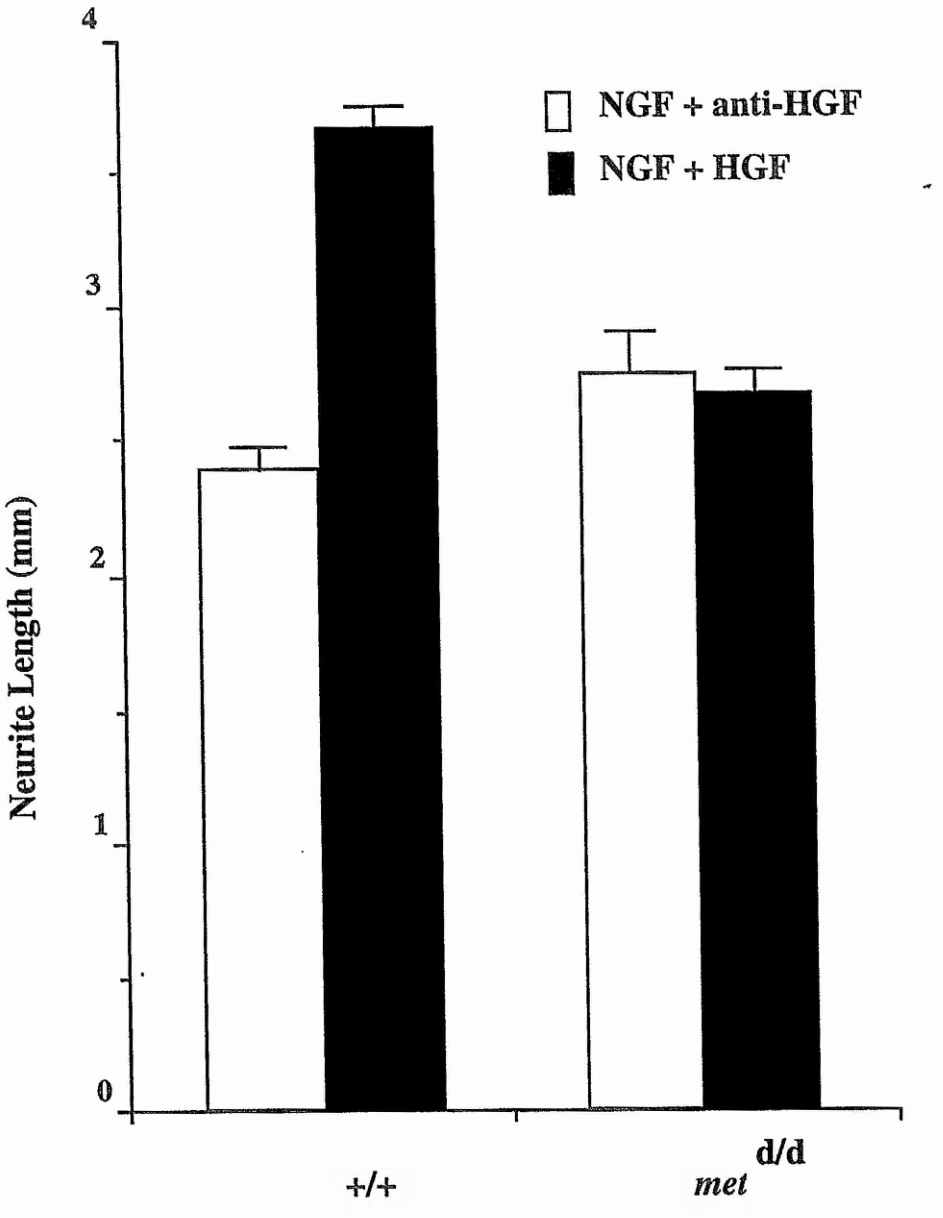


Figure 4.17: Bar graphs showing the results of neuron cohort experiments comparing neurite length of SCG from E14.5 Met-deficient and wildtype embryos after 48 hrs in culture with NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml) after 48rs in culture. The means and standard errors are shown of serial measurements made on between 50-and-100 neurons of each genotype. Data was compiled from cultures set up from the embryos of three separate litters, (+/+ n = 5, met d/d n = 7).

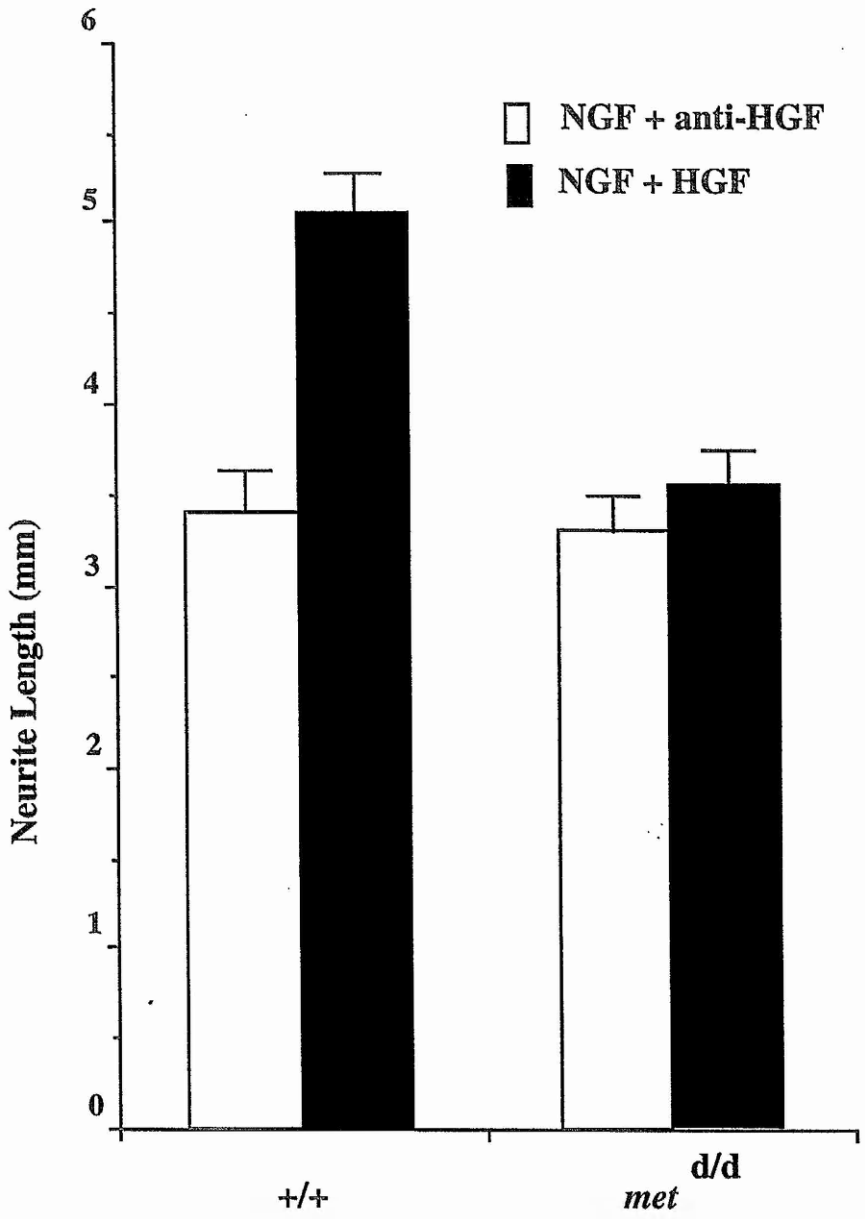


Figure 4.18: Bar graphs showing the results of neuron cohort experiments comparing neurite length axonal of TPG neurons from E14.5 Met-deficient and wildtype embryos after 48 hrs in culture with NGF (5ng/ml) plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml) after 48rs in culture. The means and standard errors are shown of serial measurements made on between 50-and-100 neurons of each genotype. Data was compiled from cultures set up from the embryos of three separate litters,(+/+ n = 5, met d/d n =7).

4.2.5 HGF cooperates with NGF to enhance neurite branching of Sympathetic neurites

To investigate the effects of HGF/Met signalling in sympathetic neurite branching, SCG (Fig. 4.19) and TPG neurons (Fig 4.20) from different stages of development (E12.5-E15.5) were cultured. After 48 hrs in culture, neurite branching was shown to be significantly greater in neurons cultured with HGF plus NGF, than neurons cultured in NGF alone or NGF plus anti-HGF at all ages studied (E12.5, E13.5, E14.5, and E15.5; $p< 0.0005$ in all cases; t tests). The difference in neurite branching was most marked in E14.5 TPG neurons which had 2-fold more branch points in the presence of HGF plus NGF than in NGF alone or NGF plus anti-HGF. Although the neurite branching in neurons cultured with anti-HGF plus NGF were generally less than those cultured with NGF alone, these differences were not significant ($p> 0.05$; t tests) in all cases except for E13.5 neurons ($p< 0.05$; t tests). After 24 hrs in culture, there was no significant difference in neurite branching between SCG and TPG neurons cultured with NGF plus HGF or anti-HGF (Figs. 4.21 and 4.22). However, between 24 and 48 hrs, neurite branching of HGF-supplemented neurons increased much more rapidly than neurons supplemented with anti-HGF resulting in significantly more neurite branching in HGF-supplemented neurons at 48 and 72 hrs ($p < 0.0001$; t test).

To determine the range of concentrations over which HGF enhances sympathetic neurite branching in vitro, E14.5 TPG and SCG neurons were cultured with or without 2ng/ml NGF over a wide range of concentrations (3.2 pg/ml-50ng/ml) (Fig. 4.23 and 4.24). For both SCG and TPG neurons the number of branch points per neurite was significantly increased in cultures containing NGF plus HGF when the concentration of HGF was at 10 ng/ml or greater ($p< 0.02$; t test). To determine the range of concentrations of NGF over which HGF enhances sympathetic neurite branching, E14.5 TPG and SCG neurons were cultured with NGF over a wide range of concentrations (Fig. 4.25 and 4.26) either with or without HGF at 10ng/ml. In TPG neurons, the number of branches per neurite

was significantly greater in HGF supplemented cultures when NGF was present at concentrations ranging from 0.4-10 ng/ml ($p< 0.005$; t test), but not at concentrations above or below this. In SCG neurons, the number of branches per neurite in HGF-supplemented cultures was significantly greater when NGF was present at concentrations ranging from 2-5 ng/ml ($p< 0.005$; t tests). These results clearly show that the neurite branching of sympathetic neurons is regulated by NGF and HGF over a wide range of concentrations and combinations.

To confirm a requirement for Met signalling in mediating the effects of HGF on neurite growth, I studied the neurite branching of sympathetic neurons of wild-type embryos and *met*$^{d/d}$ signaling mutant embryos. Whereas the neurite branching of SCG neurons (Fig. 4.27) and TPG neurons (Fig. 4.28) from wild-type embryos were significantly greater in medium containing NGF plus HGF than in medium containing NGF plus anti-HGF ($P < 0.0001$; t-tests), there was no significant difference in the average number of branche per neurite on SCG and TPG neurons from *met*$^{d/d}$ embryos grown in medium containing NGF plus HGF compared to medium containing NGF plus anti-HGF ($P > 0.05$; t-tests). Moreover, there was no significant difference in the average number of branches per neurite on neurons from *met*$^{d/d}$ embryos compared to neurons from wild-type embryos grown with NGF plus anti-HGF ($P > 0.05$ in all cases; t-tests). These results suggest that HGF/Met signalling plays a role in enhancing sympathetic neurite branching and showed that the anti-HGF does not exert a non-specific detrimental effect on neurite growth since it does not decrease neurite branching in cultures established from *met*$^{d/d}$ embryos.

4.2.6 Increased branching is a secondary effect

The increased number of branch points in the neurite arbors of sympathetic neurons may be due to a direct effect of HGF on branching itself or may be secondary to the overall increase in the size of neurite arbors in the presence of HGF. If HGF has a direct effect on branching, one would expect branch points to

occur with increased frequency with distance along axons, whereas if the increase in branch points is secondary to an overall increase in the size of neurite arbors, one would expect no difference in the intervals at which branch points occur. To distinguish between these possibilities, I measured the distance between the cell body and the first branch point in each neurite and the distance between subsequent branch points. These respective distances were not significantly different for neurons growing with NGF plus either HGF or anti-HGF in cultures of SCG and TPG established at E12.5, E13.5, E14.5 and E15.5 ($p > 0.05$ in all cases; t tests). This analysis indicates that the greater number of branch points in the arbors of neurons growing with HGF is secondary to the effect of HGF on enhancing axonal growth.

4.2.7 HGF increases the number or neurites

Although HGF does not have a direct effect on increasing the frequency with which an axon branches, it does increase the number of neurites that emerge from the cell bodies of cultured sympathetic neurons. In cultures set up from TPG (Fig. 4.29) neurons, there were more unipolar neurons in cultures supplemented with NGF plus anti-HGF than in cultures supplemented with NGF alone or with NGF plus HGF. Conversely there were more tripolar neurons in cultures supplemented with NGF plus HGF than in cultures supplemented with NGF alone or with NGF plus anti-HGF. The typical appearances of E14.5 TPG neurons grown for 48 hrs with NGF plus anti-HGF and NGF plus HGF are shown in figure 4.30.

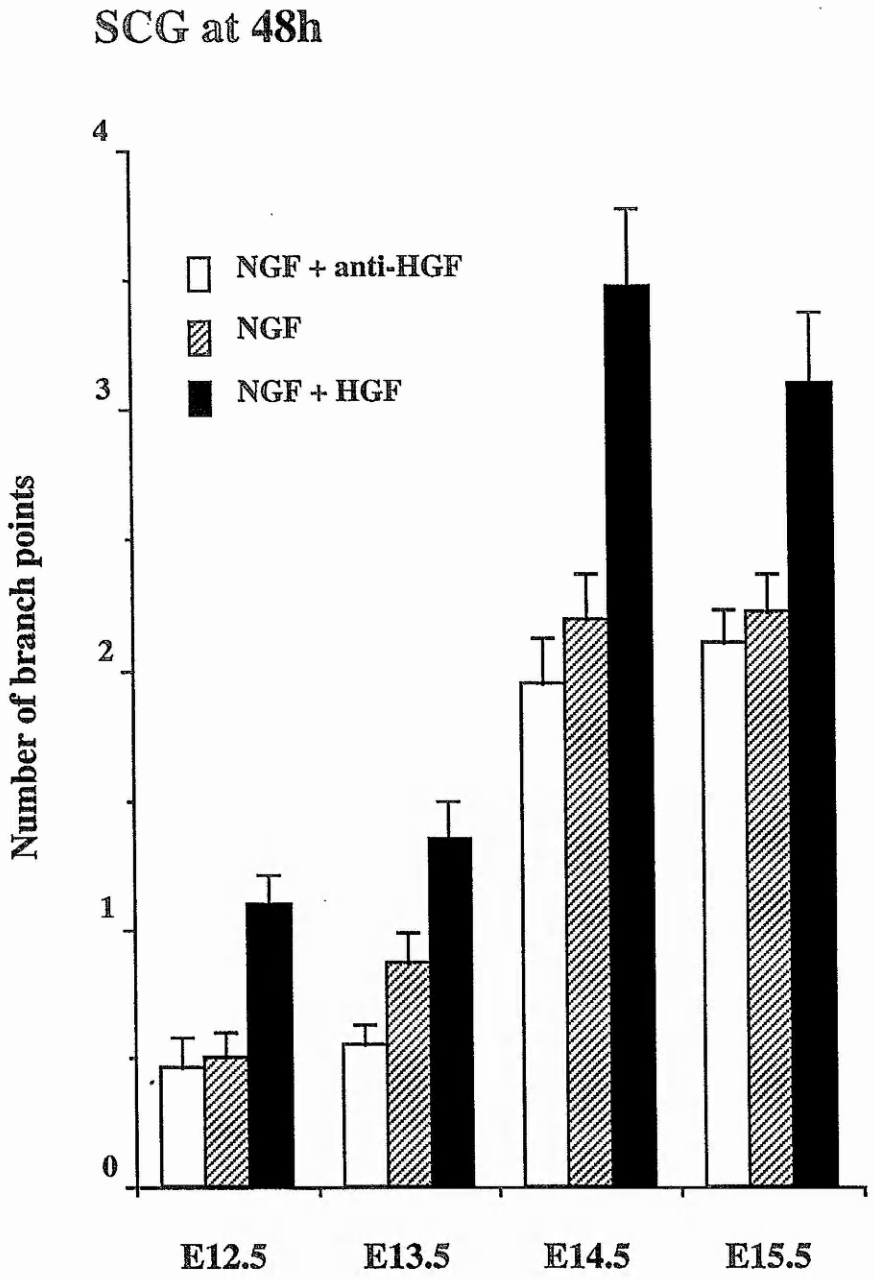


Figure 4.19: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of SCG neurons from different aged embryos after 48 hrs in culture in NGF (5ng/ml), NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml) after 48hrs in culture. The means and standard errors are shown of serial measurements made on between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

TPG at 48h

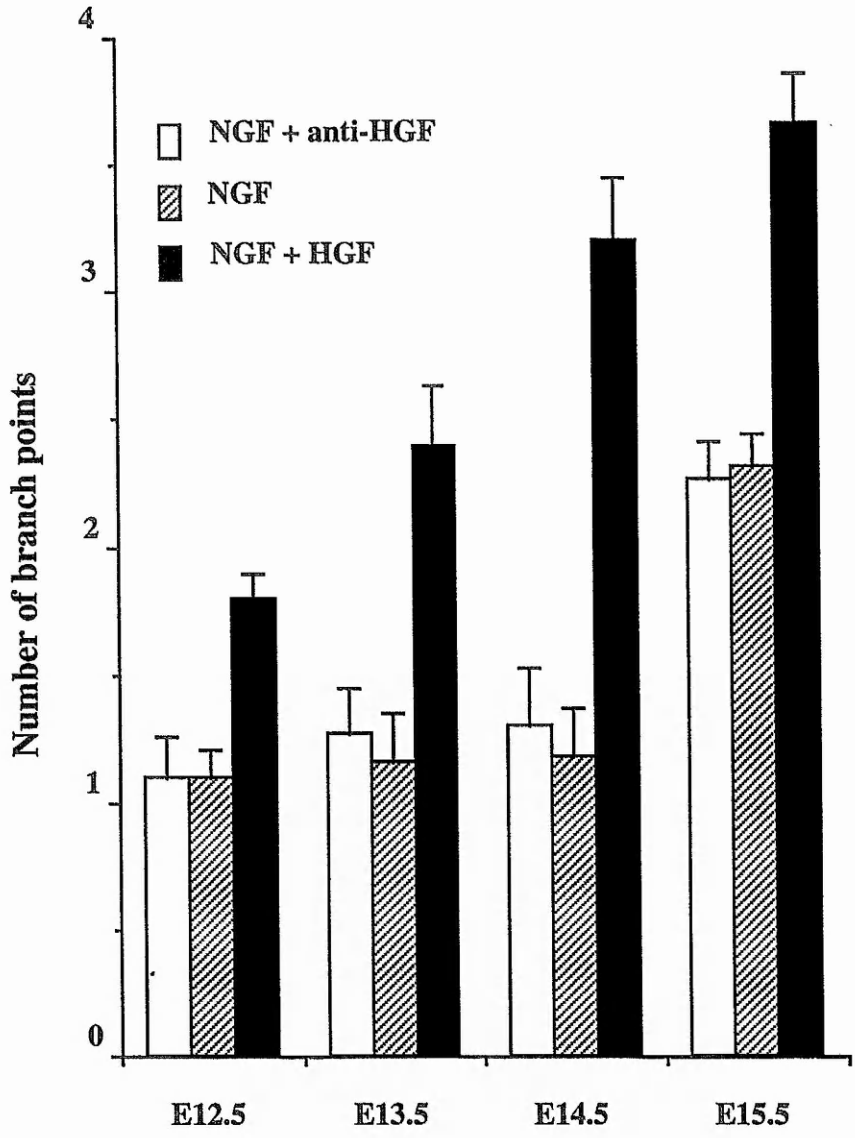


Figure 4.20: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of TPG neurons from different aged embryos after 48 hrs in culture with NGF (5ng/ml) NGF plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

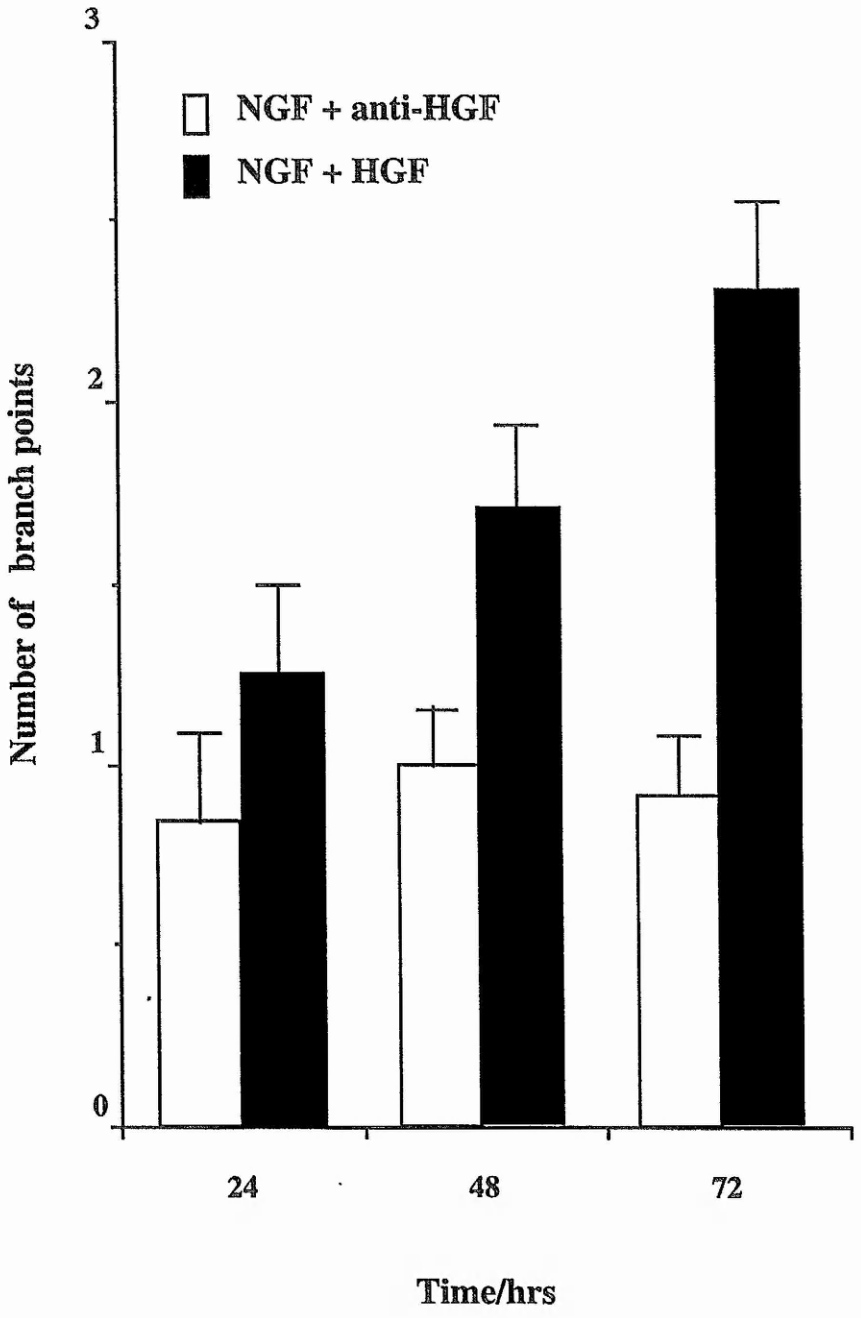


Figure 4.21: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of SCG neurons from E14.5 embryos over 72 hrs in cultures NGF (5ng/ml) plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of from between between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments) .

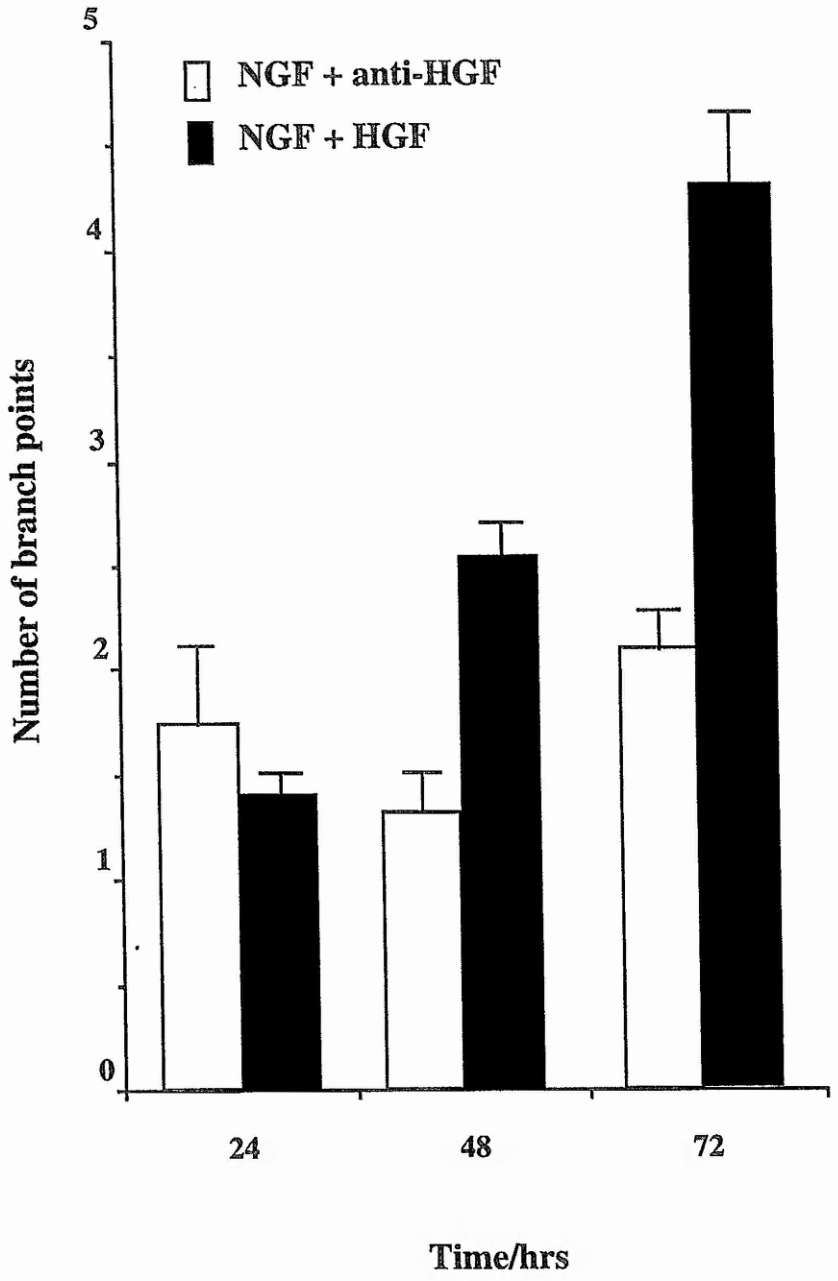


Figure 4.22: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of TPG neurons from E14.5 embryos over 72 hrs in culture with NGF (5ng/ml) plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

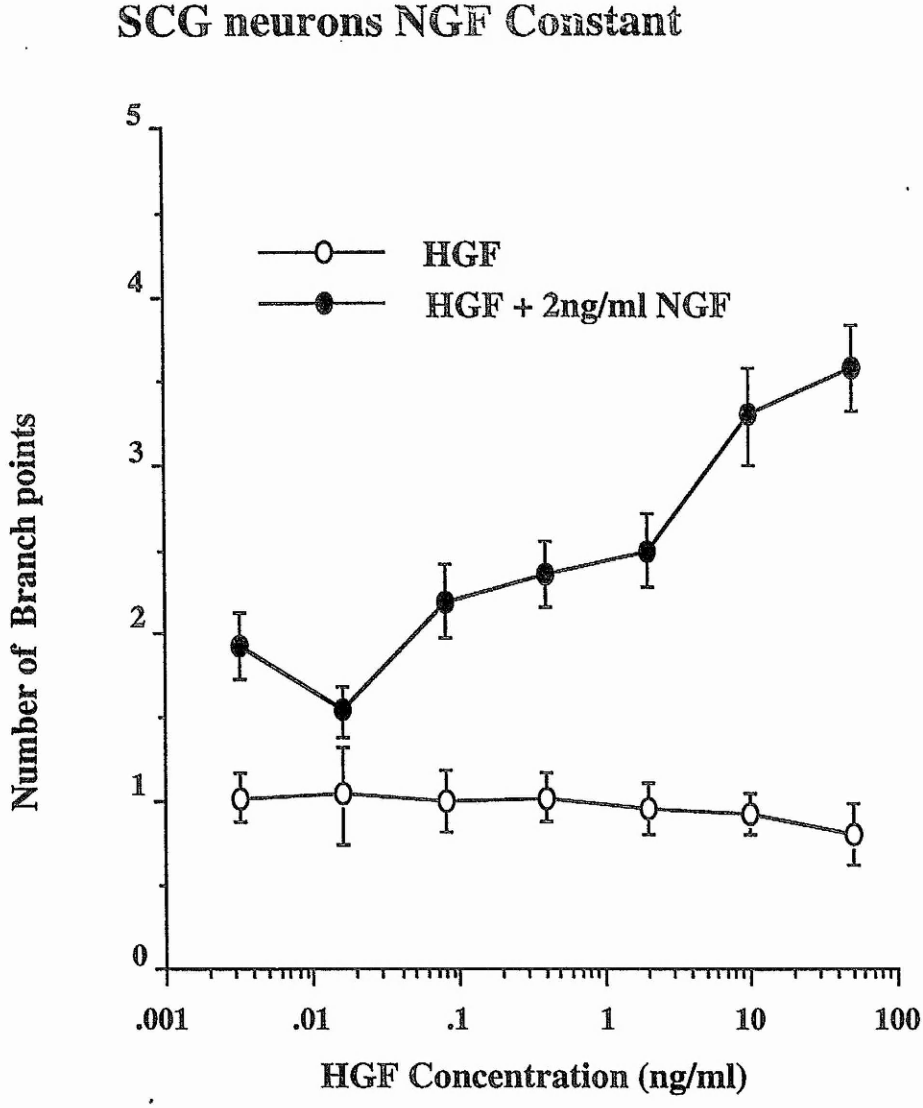


Figure 4.23: Dose response of the HGF effect on E14.5 SCG neurons. The graph shows the number of branches after 48 hrs in culture. Neurons were cultured with a range of HGF concentrations either with or without 2ng/ml. NGF The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments)

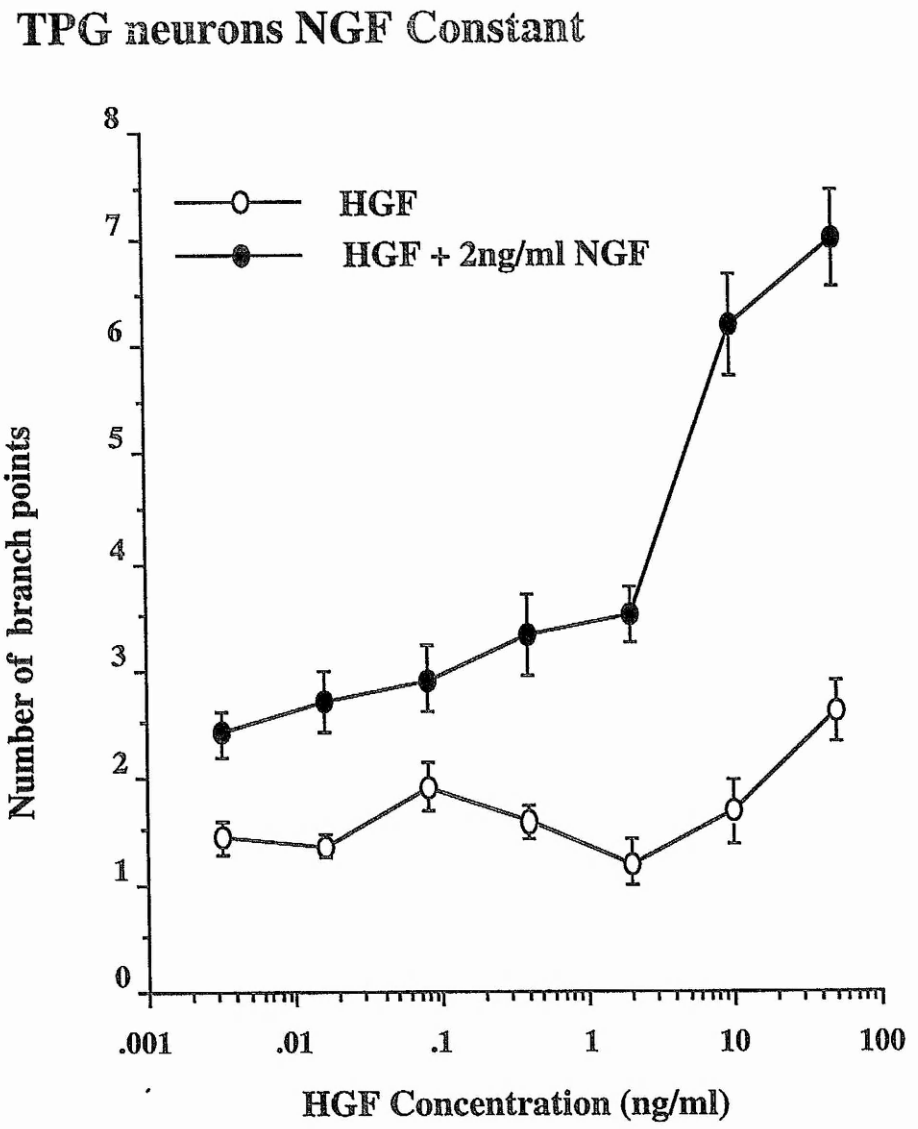


Figure 4.24: Dose response of the HGF effect on E14.5 TPG neurons. The graph shows the number of branches per neurite after 48 hrs in culture. Neurons were cultured with a range of HGF concentrations either with or without 10ng/ml NGF. The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

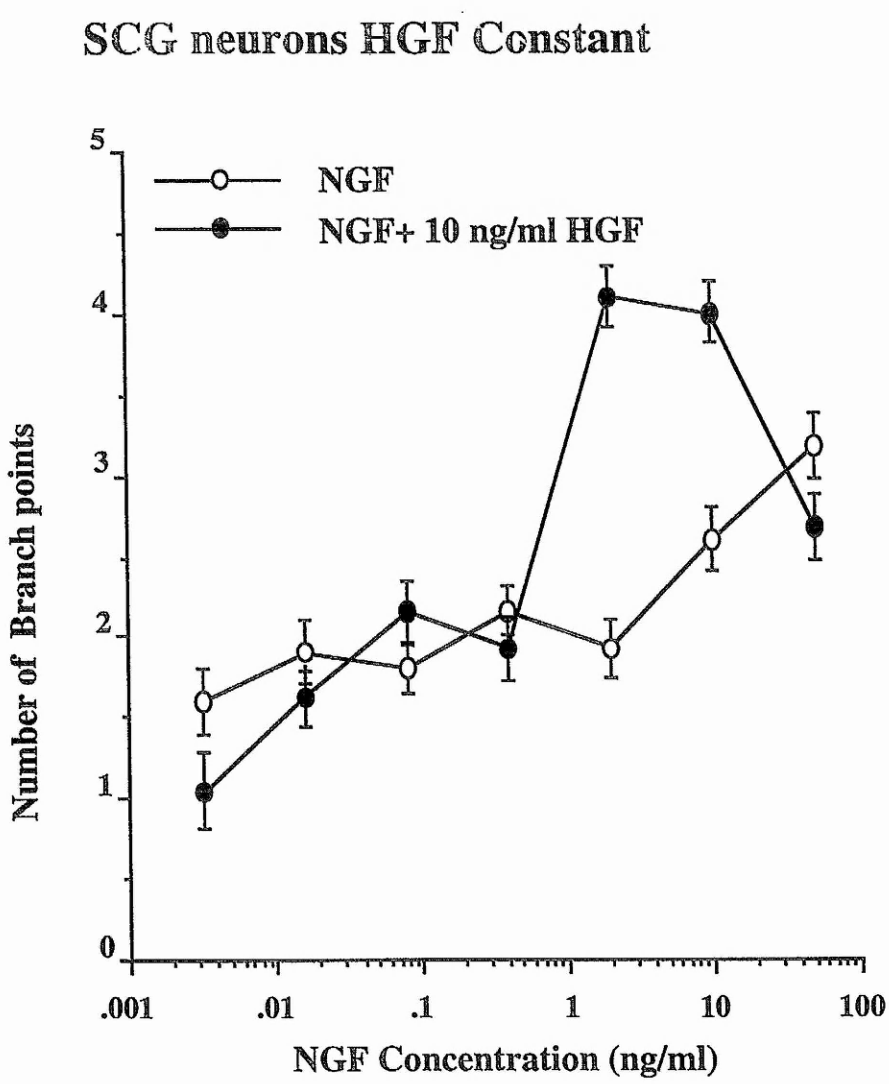


Figure 4.25: Dose response of the NGF effect on E14.5 SCG neurons. The graph shows the number of branches per neurite after 48 hrs in culture. Neurons were cultured with a range of NGF concentrations either with or without 10ng/ml HGF The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

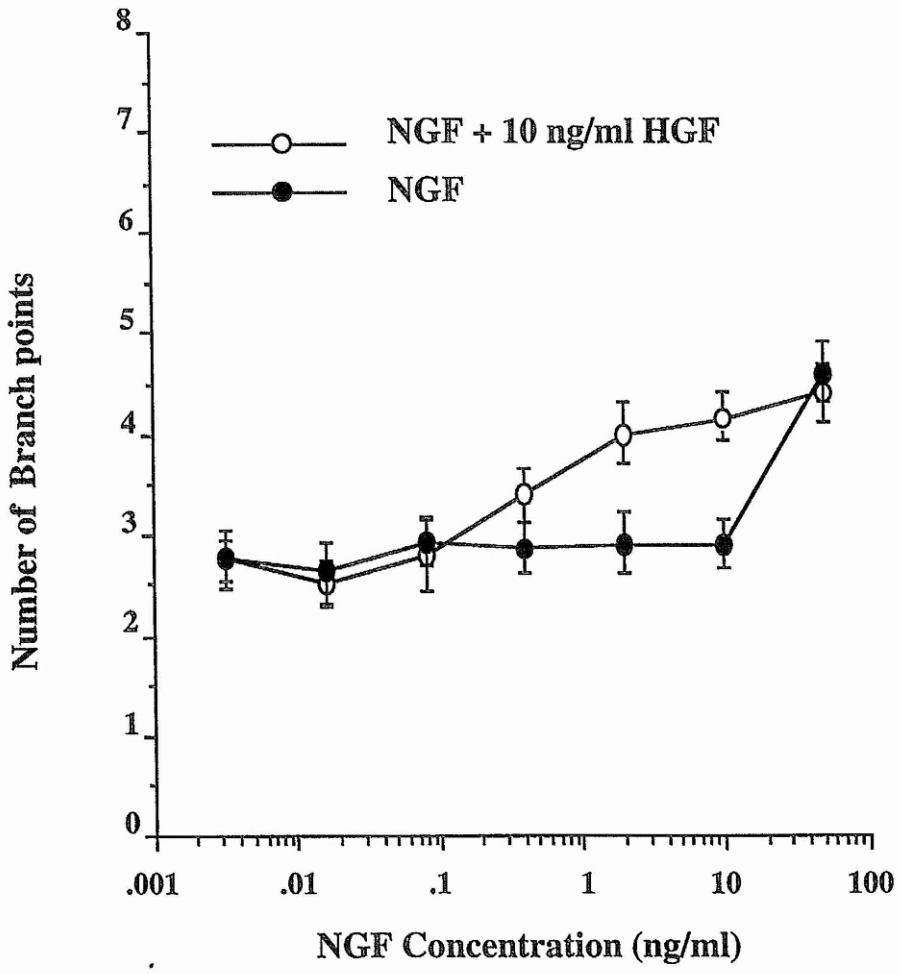


Figure 4.26: Dose response of the NGF effect on E14.5 TPG neurons. The graph shows the number of branches per neurite after 48 hrs in culture. Neurons were cultured with a range of NGF concentrations either with or without 10ng/ml HGF. The means and standard errors are shown of counts taken from between 70 and 122 neurons in each experimental group (compiled from cultures set up from three separate experiments).

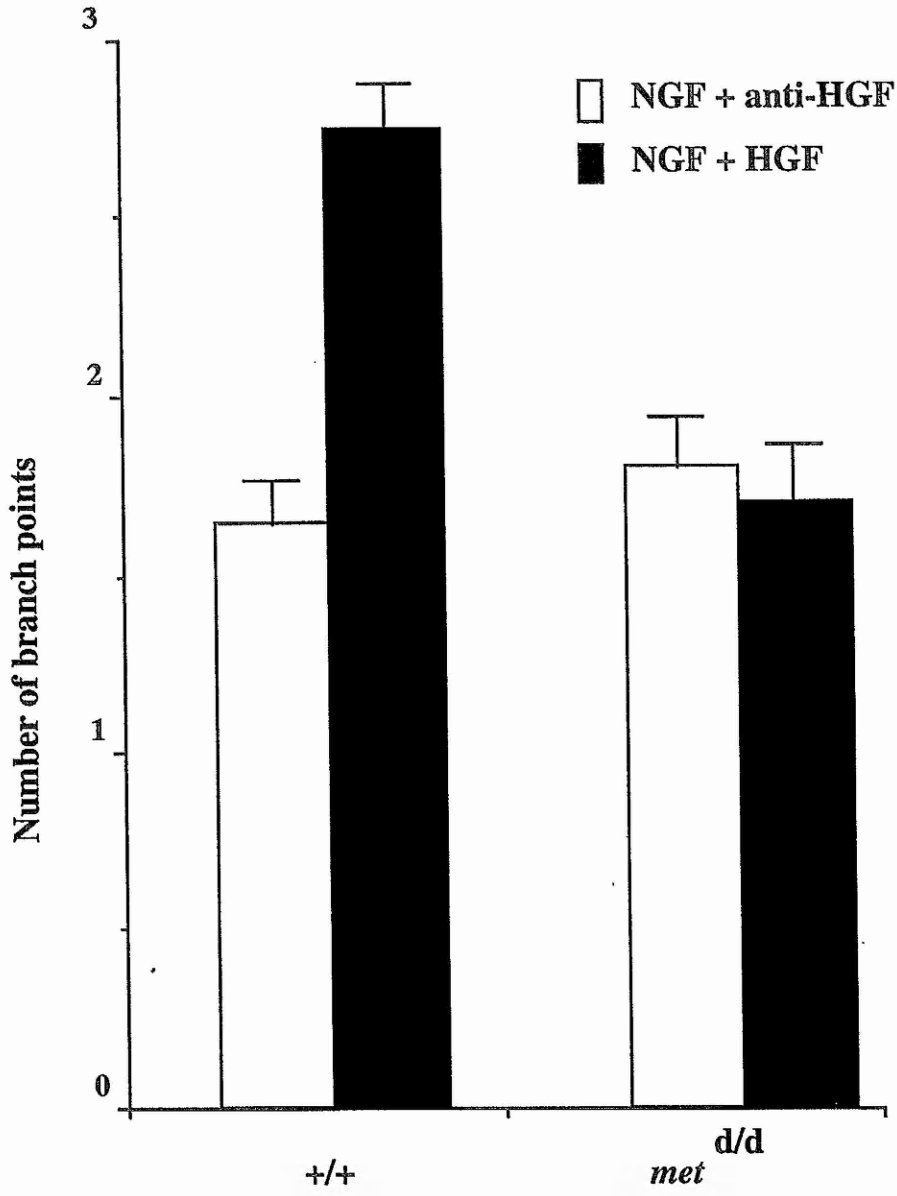


Figure 4.27: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of SCG neurons from E14.5 Met-deficient and wildtype embryos after 48 hrs in culture with NGF (5ng/ml) plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml. The means and standard errors are shown of counts taken from between 50-and- 150 neurons of each genotype. The data is compiled from cultures set up from the embryos of three separate litters, +/+ n = 5, met d/d n = 7

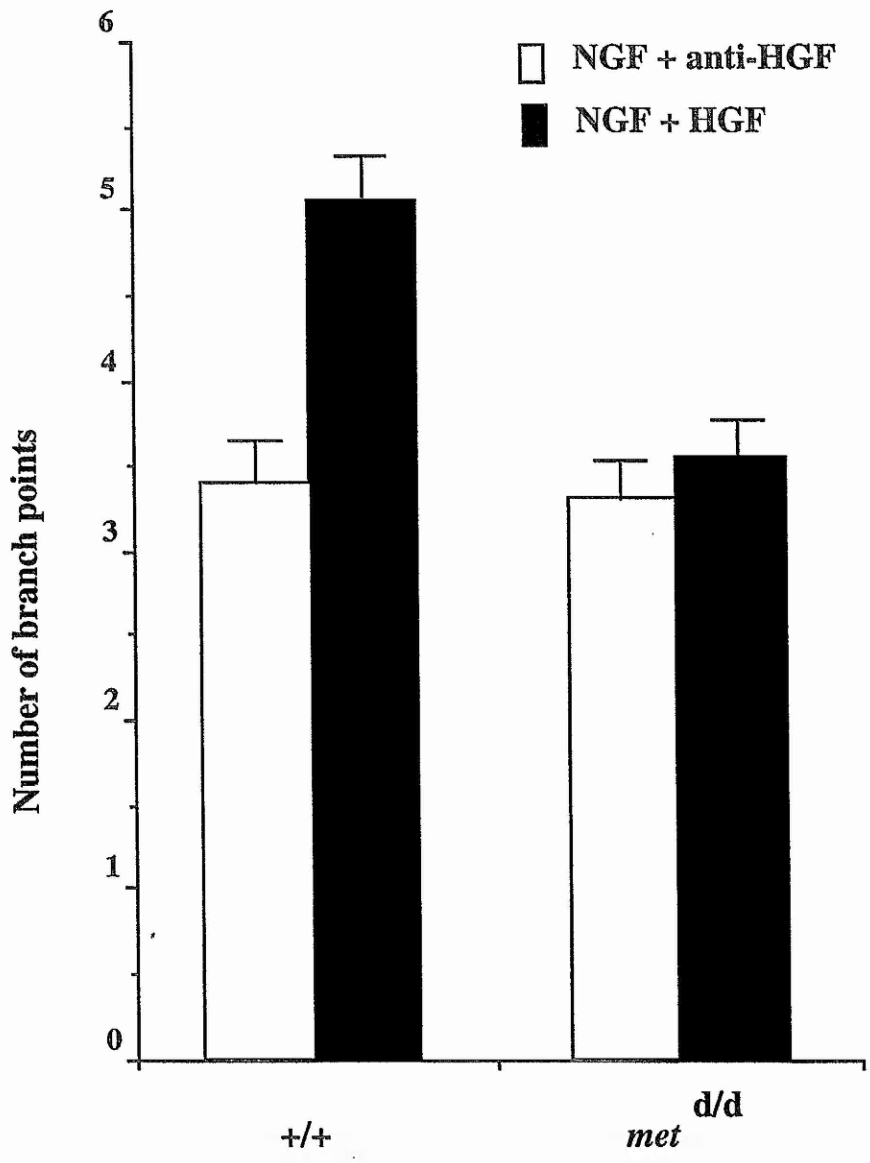


Figure 4.28: Bar graphs showing the results of neuron cohort experiments comparing neurite branching of TPG neurons from E14.5 Met-deficient and wildtype embryos after 48 hrs in culture with NGF (5ng/ml) plus anti-HGF (1.25 mg/ml), or NGF plus HGF (10ng/ml). The means and standard errors are shown of counts taken from between 50-and-150 neurons of each genotype. The data is compiled from cultures set up from the embryos of three separate litters, +/+ n = 5, met d/d n = 7.

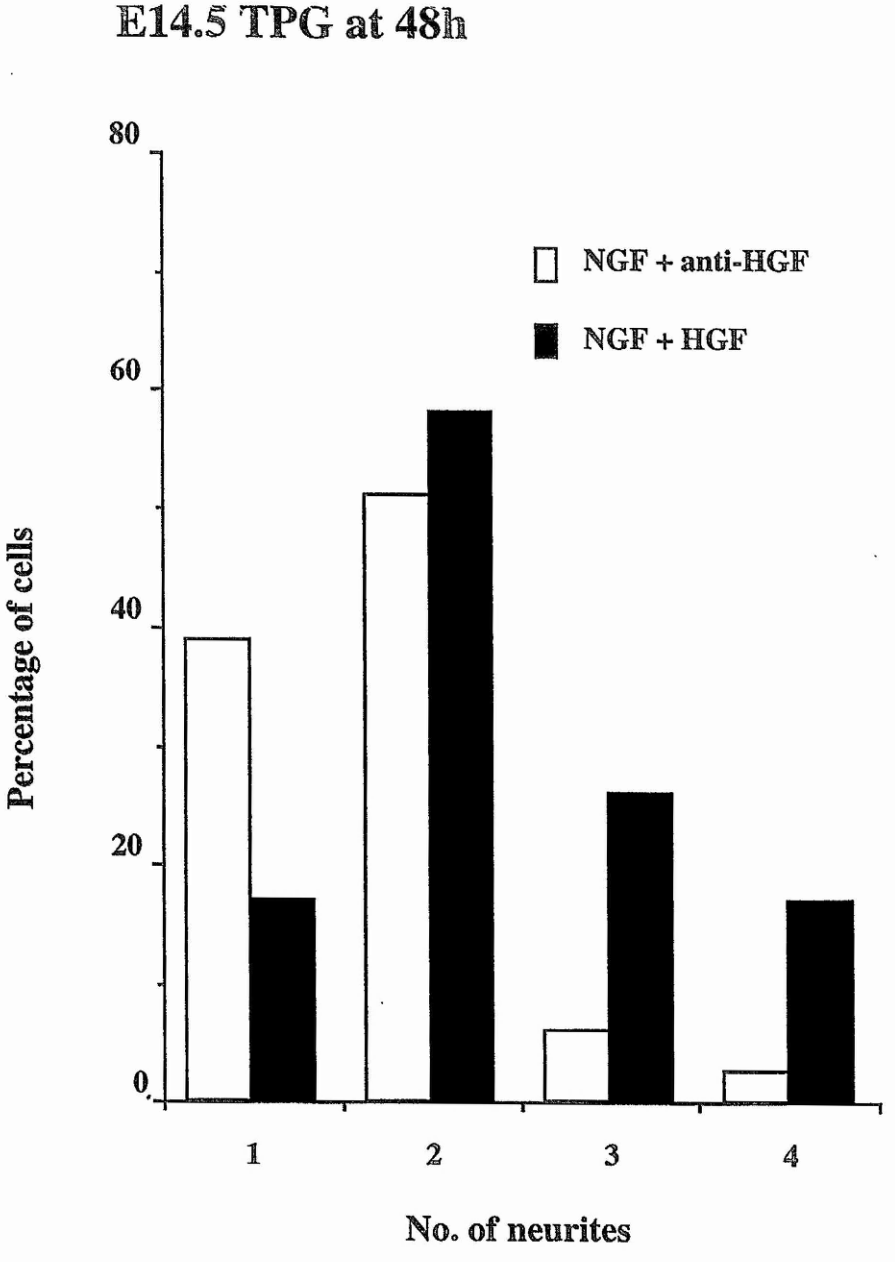


Figure 4.29: Bar graph showing the percentage of TPG neurons possessing one, two, three. Or four neurites after 48 hrs in culture with either NGF plus HGF or NGF plus anti-HGF.

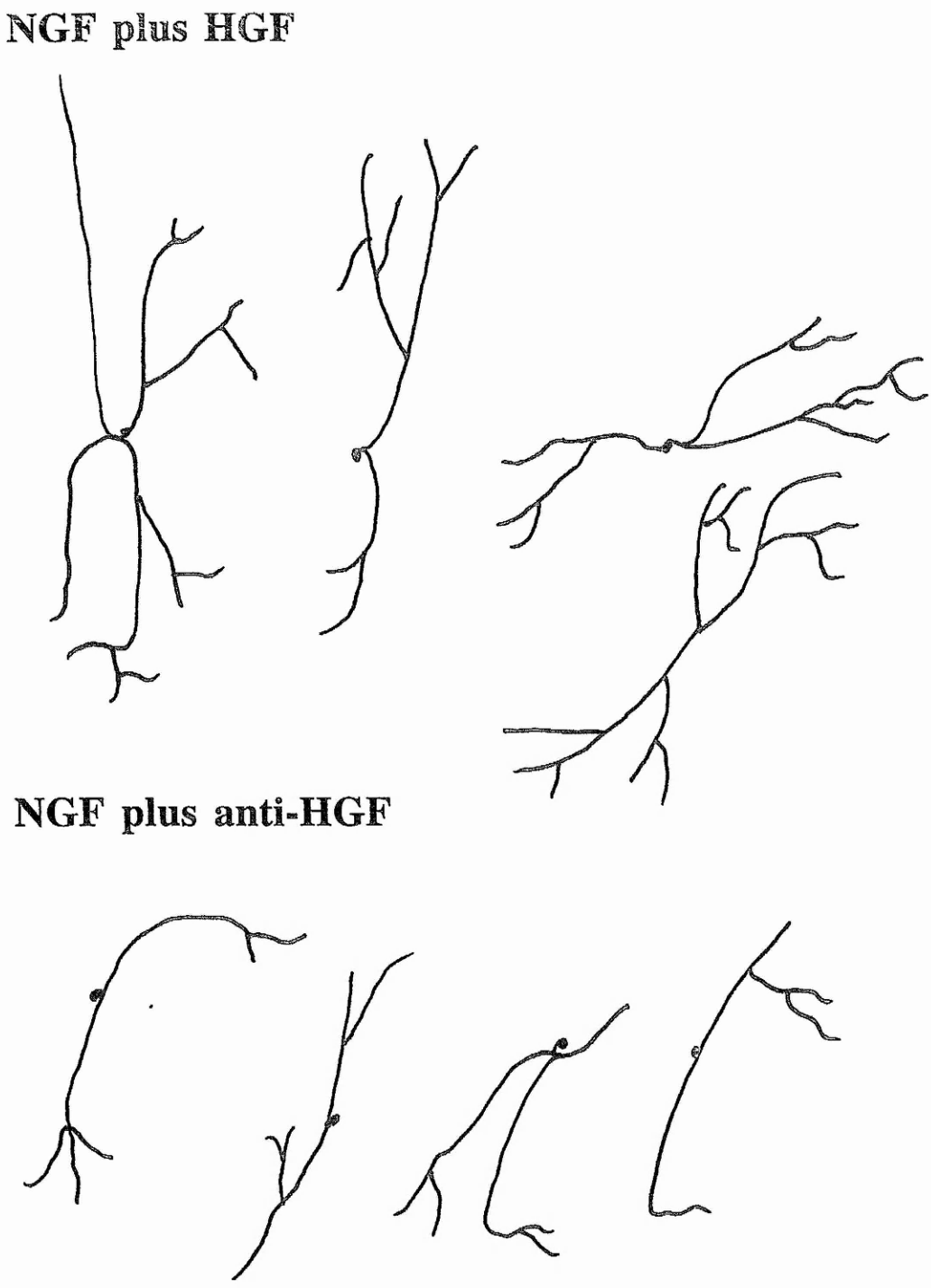


Figure 4.30: Camera lucida drawings Typical appearance of E14.5 sympathetic neurons grown for 48 hours with either NGF plus anti-HGF or NGF plus HGF. (Scale Bar = 2mm).

4.3 Discussion

The experimental studies described in this chapter demonstrate that HGF/Met signalling exerts a variety of specific effects on the development of sympathetic neuroblasts and neurons of the paravertebral sympathetic chain both in vitro and in vivo. Experiments in which the fates of individual cells were carefully followed over time in culture clearly demonstrated that HGF/Met signaling affects sympathetic neuroblast differentiation and survival. Consistent with these data, in the early SCG of homozygous *met*$^{d/d}$ mutant embryos there is an increased proportion of neuroblasts undergoing apoptosis, and a progressive reduction in the total number of cells.

Anti-HGF reduced the number of neuroblasts that differentiated into neurons in culture and increased the number of neuroblasts that died before differentiating. Both effects of anti-HGF were not observed in neuroblasts obtained from *met*$^{d/d}$ embryos, indicating that Met signalling is required for these effects and that anti-HGF does not exert a non-specific detrimental effect on the survival and differentiation of progenitor cells. Because HGF and Met are expressed in sympathetic ganglia from the earliest stages of their development and Met protein is expressed by neuroblasts and neurons in early sympathetic ganglia, it is likely that neuroblast survival and differentiation is enhanced by HGF produced by the cells themselves. Interestingly, added HGF did not further enhance neuroblast survival and differentiation in these cultures, suggesting that endogenously produced HGF is maximally effective in enhancing neuroblast survival and differentiation. Endogenously produced HGF may act by an autocrine or paracrine mode on neuroblasts in early sympathetic ganglia. Although it was not possible to distinguish between these two modes of action in the culture system used in these studies, the fact that the effects of anti-HGF were observed in extremely low density cultures would favour an autocrine mode.

An autocrine role for the neurotrophin BDNF in the early stages of neuronal differentiation has previously been reported in early dorsal root ganglion sensory

neurons which express BDNF mRNA *in vivo* (Wright et al., 1992). During the earliest stages of their development, these neurons undergo a clearly recognizable morphological change. Initially, they have small, spindle-shaped, phase-dark cell bodies and short neurites and subsequently develop spherical, phase-bright cell bodies and extend long neurites. This change, which takes place in single cell cultures, is accelerated by BDNF and retarded by antisense BDNF oligonucleotides. These antisense oligonucleotides do not, however, affect the survival of early DRG neurons, suggesting that the BDNF autocrine loop is not required for survival at this stage (Wright et al., 1992). Similar experiments with antisense BDNF oligonucleotides carried out on adult DRG neurons suggest that a BDNF autocrine loop maintains the survival of a subset of these older neurons (Acheson et al., 1995). There is some evidence that another neurotrophin, NT-3, may be required for the survival of sensory neuron progenitor cells. It has been reported that the majority of cells undergoing apoptosis in the early DRG of *NT-3*$^{-/-}$ embryos (recognized by TUNEL labelling) had also incorporated BrdU administered 5 hours earlier (ElShamy and Ernfors, 1996). However, in another study, no BrdU-positive/TUNEL-positive cells were observed in the early DRG of *NT-3*$^{-/-}$ embryos, although BrdU-positive/neurofilament-positive cells were observed (Farinas et al., 1997).

Previous studies have shown that HGF is effective on its own in promoting the survival of cultured motoneurons (Ebens et al., 1996; Wong et al., 1997; Yamamoto et al., 1997) and that HGF cooperates with NGF, but not with BDNF or NT3, in enhancing the survival of DRG sensory neurons *in vitro* (Chapter 3). In contrast, although I have implicated endogenous HGF in enhancing the survival of sympathetic neuroblasts, the survival of differentiated sympathetic neurons is unaffected by anti-HGF and is not enhanced by HGF alone. Furthermore, HGF does not enhance the survival of sympathetic neurons growing in the presence of NGF.

I observed that HGF increased the number of neurites that grew from the cell bodies and increased the overall length and number of branch points in the neurite arbors of sympathetic neurons. Analysis of the distance between branch points indicated that the increase in the number of branch points in the presence of HGF was secondary to the increase in the overall size of the neurite arbors. All these effects of HGF are dependent on Met signalling. In contrast to the lack of effect of added HGF on neuroblast survival and differentiation, the largest changes in neurite growth and branching were observed in cultures supplemented with HGF (as opposed to those treated with anti-HGF). This suggests that HGF from sources other than the neurons themselves has the potential to regulate sympathetic axon growth and branching *in vivo*.

The studies reported in Chapter 3 showed that HGF enhances neurite outgrowth from DRG neurons cultured in the presence of NGF but not when these neurons are grown with BDNF or NT3. It is interesting to note that the HGF effects described in this and the present chapter occur in the population of sensory and sympathetic neurons which are preferentially dependent on NGF, but not on the sensory neurons located in the nodose ganglia that are preferentially dependent on BDNF. This observation indicates that the effects of HGF are not attributable to generalized increased fitness of sensory and sympathetic neurons. It would be particularly interesting to understand how these synergism's occur in terms of signaling pathways. Are Met and TrkA activating different pathways, or enhancing the level of activation of one or more pathways, or do they have a synergistic effect on the kinetics of activation? Additional Met signaling mutants will help to further define this cooperation.

In summary, this study shows multiple effects of HGF/Met signalling on sympathetic neuroblasts and neurons. HGF regulates differentiation and survival of sympathetic neuroblasts and has the potential to enhance axonal growth of NGF-dependent sympathetic neurons. This suggests that HGF can act in an autocrine and

paracrine manner to regulate sympathetic neuron development during embryogenesis.

Chapter 5
Co-operation between HGF and CNTF in promoting the survival and growth of sensory and parasympathetic neurons

5.1 Introduction

Previous *in vitro* studies described in chapters 3 and 4 of this thesis have shown that HGF promotes the survival and differentiation of sympathetic neuroblasts and that in the presence of NGF, but not other neurotrophins, HGF enhances the survival and growth of a subset of DRG neurons and enhances the growth of sympathetic neurites. To investigate if HGF influences the survival and growth of other classes of neurons in the developing peripheral nervous system and synergises with neurotrophic factors other than NGF, I studied its effect on purified cultures of parasympathetic neurons of the ciliary ganglion and proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN). The survival of the great majority of neurons in both populations is supported by CNTF in culture (Barbin et al., 1984; Allsopp et al., 1993). The survival of TMN is also supported just as effectively by BDNF (Davies et al., 1986a, 1986b; Allsopp et al., 1995; Davey & Davies, 1998). NGF does not, however, promote the survival of either ciliary (Helfand et al., 1976; Rohrer & Sommer, 1982) or TMN neurons (Davies et al., 1987). The results presented in this chapter show that whilst HGF alone does not promote the survival of either of these kinds of neurons on its own, it does increase the number of neurons surviving in cultures supplemented with CNTF, but not BDNF. HGF was also shown to increase the overall length and branching of the neurite arbors of these neurons in the presence

of CNTF, but not BDNF. These results show that HGF is able to co-operate with CNTF in promoting the survival and growth of developing parasympathetic and proprioceptive neurons. The demonstration that HGF only enhanced the survival and growth of TMN neurons when these neurons were grown with CNTF and not when they were grown with BDNF (which promoted their survival as effectively as CNTF), demonstrates that within the same neurons the effects of HGF on survival and growth are selectively dependent on which other signalling pathways are concurrently activated.

5.2 Results

5.2.1 Survival

To investigate if HGF influences the survival of ciliary and TMN neurons, I established low density dissociated cultures of these neurons from embryonic day (E10) chicken embryos, a stage when naturally occurring neuronal death is taking place in these populations of neurons *in vivo* (Landmesser & Pilar, 1974; Rogers & Cowen, 1974). To ensure that only direct effects of HGF on neurons were observed, differential sedimentation (Davies, 1986) was used to remove almost all non-neuronal cells from these cultures prior to plating (> 95% pure neurons). These neurons were grown with HGF alone or HGF plus other neurotrophic factors that are known to promote the survival of these neurons (CNTF or BDNF) and the proportion of neurons that survived in these cultures and control cultures (no added factors) was determined after 48 hours incubation.

5.2.2 Ciliary Neurons

HGF over a broad range of concentrations (from 3.2 pg/ml to 250 ng/ml) had no effect on the survival of ciliary neurons on its own (Fig. 5.1). The small number of neurons surviving in these cultures (approximately 5% after 48 hours) was not significantly different from the number in control cultures ($p > 0.05$, t-tests). CNTF was very effective in promoting the survival of ciliary neurons (Fig. 5.2). Dose response analysis revealed that at saturating concentrations (50 ng/ml and above) approximately 60% of the neurons were still surviving after 48 hours. Although HGF did not support the survival of ciliary neurons on its own, several experiments showed that HGF in combination with CNTF promoted the survival of more neurons than CNTF alone. To ascertain the concentration at which HGF begins to enhance the survival of ciliary neurons incubated with CNTF, ciliary neurons were grown with a

constant saturating level of CNTF (50 ng/ml) plus HGF over a range of concentrations (Fig. 5.1). The number of neurons growing with CNTF plus HGF at concentrations at and above 2 ng/ml was significantly greater than the number surviving with CNTF alone ($p < 0.01$, t tests). At the highest concentration of HGF used (250 ng/ml) there was a 50% increase in neuronal survival compared with CNTF alone. Very similar results were obtained when CNTF was used at a concentration of 5 ng/ml (data not shown). To ascertain the concentration range of CNTF over which HGF enhances survival, ciliary neurons were grown with a constant level of HGF (2 ng/ml) plus CNTF over a broad range of concentrations (Fig 5.2). Neuronal survival in HGF supplemented cultures was significantly greater than in cultures containing CNTF alone at concentrations of CNTF at and above 2 ng/ml ($p < 0.05$, t test). Very similar results were obtained when HGF was used at a constant concentration of 10 ng/ml (data not shown). These results demonstrate that although HGF does not promote ciliary neuron survival alone, it co-operates with CNTF in enhancing the survival of these neurons over a particular combination of concentrations.

5.2.3 TMN Neurons

Figure 5.3 shows that the survival of the majority of E10 TMN neurons was promoted by either BDNF or CNTF alone. HGF alone, however, did not promote the survival of these neurons (there was no significant difference between the small number of neurons surviving in control cultures and those containing HGF ($p > 0.05$, t test). However, there was a significant 57% increase in the number of neurons in cultures containing CNTF plus HGF compared with cultures containing CNTF alone ($p < 0.001$, t test). In contrast, there was no significant difference between the number of neurons surviving in cultures containing HGF plus BDNF compared with cultures containing BDNF alone ($p > 0.05$, t test). The concentrations of CNTF and BDNF used in the present studies (10 ng/ml) have been shown to be maximally effective for

promoting TMN survival (Davies et al., 1986a; Davey & Davies, 1998). Because only very limited numbers of purified TMN neurons were available from each embryo, it was not feasible to undertake the detailed dose response analysis for different concentrations of CNTF and HGF that were undertaken for ciliary neurons. However, separate experiments in which HGF was used at a concentration of 50 ng/ml also specifically enhanced the survival responses of TMN neurons to CNTF but not to BDNF (data not shown).

5.2.4 Axonal Growth

To investigate if HGF affects the growth of neurites from ciliary and TMN neurons, drawings were made of neurons grown with CNTF alone or CNTF plus HGF (additionally, TMN neurons were also grown with BDNF alone or BDNF plus HGF). The total length of each neurite arbor was measured at intervals by digitising the drawings. To avoid any confusion about which neurites emanated from each cell body, the neurons were grown at very low density so that their neurite arbors did not overlap. To avoid the effects of HGF on neuronal survival biasing the data, serial drawings were made of the same neurons between 3 hours and 72 hours (Davies, 1989) and only neurons that survived throughout this period were included in the analysis (Hiltòn et al., 1997).

5.2.5 Ciliary neurons

After 48 hours in culture, the lengths of the neurite arbors of ciliary neurons grown with HGF plus CNTF were significantly greater than those of neurons grown with CNTF alone (Fig. 5.4). To ascertain the concentration at which HGF begins to enhance the growth of neurites incubated in the presence of CNTF, E10 ciliary neurons were grown with a constant level of CNTF (50 ng/ml) plus HGF over a wide range of concentrations (Fig. 5.4). The neurite arbors of NGF-supplemented neurons were

significantly longer in the present of HGF at concentrations of 2 ng/ml and greater ($p < 0.005$ at this concentration; t-test), but not at lower concentrations. To ascertain the concentration range of CNTF over which HGF enhances neurite growth, ciliary neurons were grown with a constant level of HGF (2 ng/ml) plus CNTF over a wide range of concentrations (Fig. 5.5). The neurite arbors of HGF-supplemented neurons were significantly longer in the presence of CNTF at concentrations of 2 ng/ml and greater ($p < 0.001$ at this concentration; t-test) but not at lower concentrations. These results indicate that CNTF and HGF co-operate in regulating neurite growth over a particular combination of concentration.

In addition to enhancing the overall length of the neurite arbors emanating from developing parasympathetic neurons, HGF increased the number of branch points in the neurite arbors of these neurons. After 48 hours in culture, the number of branch points in the neurite arbors of ciliary neurons grown with CNTF plus HGF were significantly greater than those of neurons grown with CNTF alone. Figure 5.6 and 5.7 show that increased branching was observed over similar concentration ranges of CNTF and HGF to those that enhanced the overall length of the neurite arbors of ciliary neurons (Fig. 5.4 and Fig.5.5). The typical appearance of E10 ciliary neurons grown for 48 hrs with CNTF and CNTF plus HGF are shown in Figure 5.10

5.2.6 TMN neurons

Figure 5.8 shows that after 48 hours in culture, the lengths of the neurite arbors of TMN neurons grown with HGF plus CNTF were significantly greater than those of neurons grown with CNTF alone ($p < 0.0001$, t-test). Likewise, there were significantly more branch points in the neurite arbors of TMN neurons grown with HGF plus CNTF compared neurons grown with CNTF alone ($p < 0.0001$, t-test) (Fig.5.9). In contrast, there was no significant difference between the overall length and number of branch points in the neurite arbors of TMN neurons grown with HGF

plus BDNF compared with neurons containing BDNF alone ($p > 0.05$, for both length and branch points, t tests). These results show that HGF specifically synergises with CNTF but not BDNF in enhancing the growth of TMN neurites. The typical appearances of E10 TMN neurons grown for 48 hrs are shown in figure 5.11.

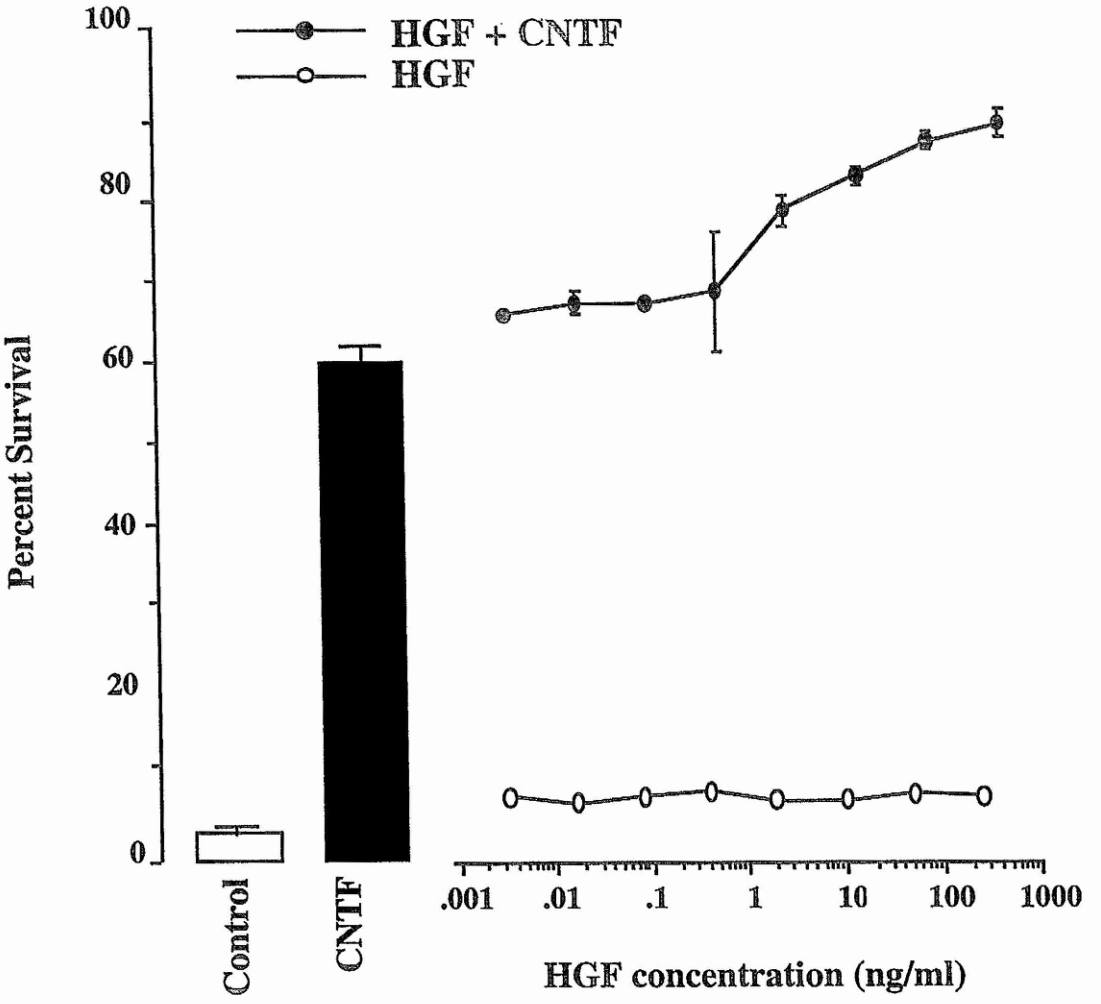


Figure 5.1: Dose responses showing the effect of HGF and CNTF plus HGF on the survival of E10 ciliary ganglion neurons after 48 hours in culture. The graph shows the percent survival in control cultures, cultures containing CNTF alone (50 ng/ml) and cultures containing HGF over a range of concentrations with or without CNTF (50 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Three additional separate experiments gave very similar results.

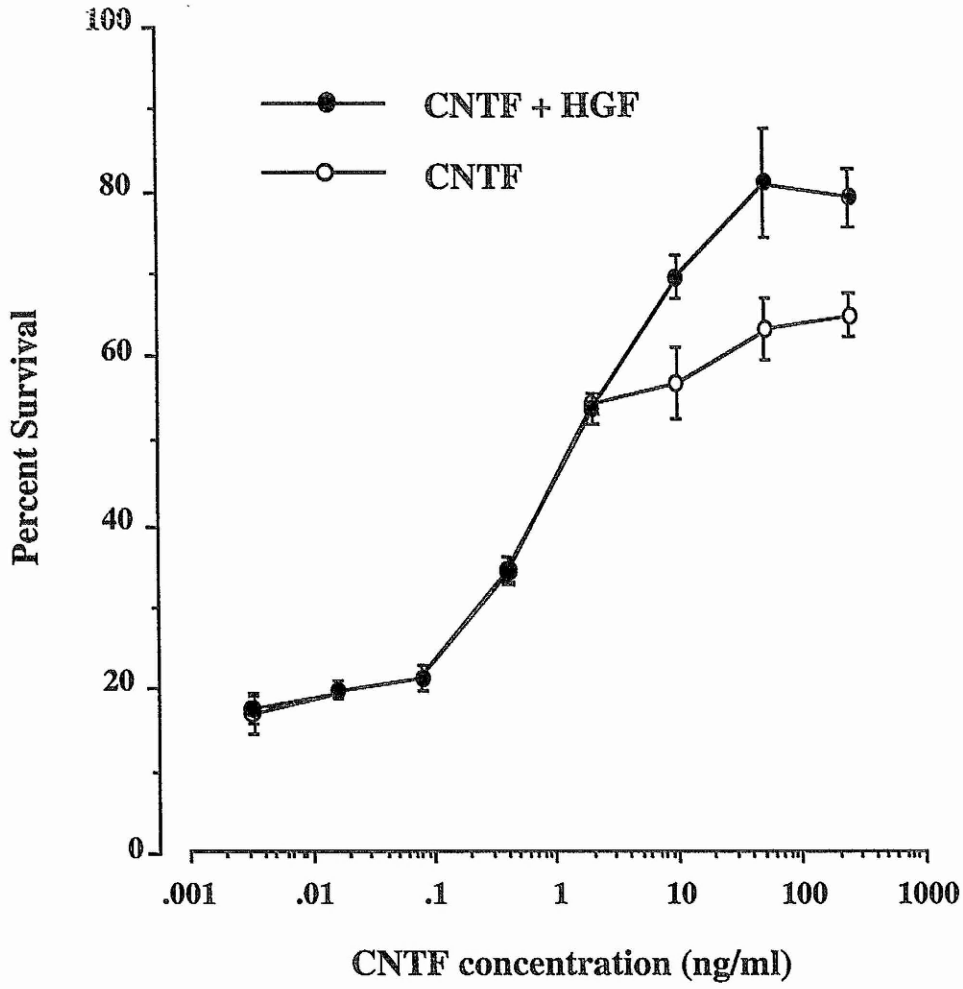


Figure 5.2: Dose responses showing the effect of HGF and CNTF plus HGF on the survival of E10 ciliary ganglion neurons after 48 hours in culture. The graph shows the percent survival in cultures containing CNTF over a range of concentrations with or without HGF (2 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Three additional separate experiments gave very similar results.

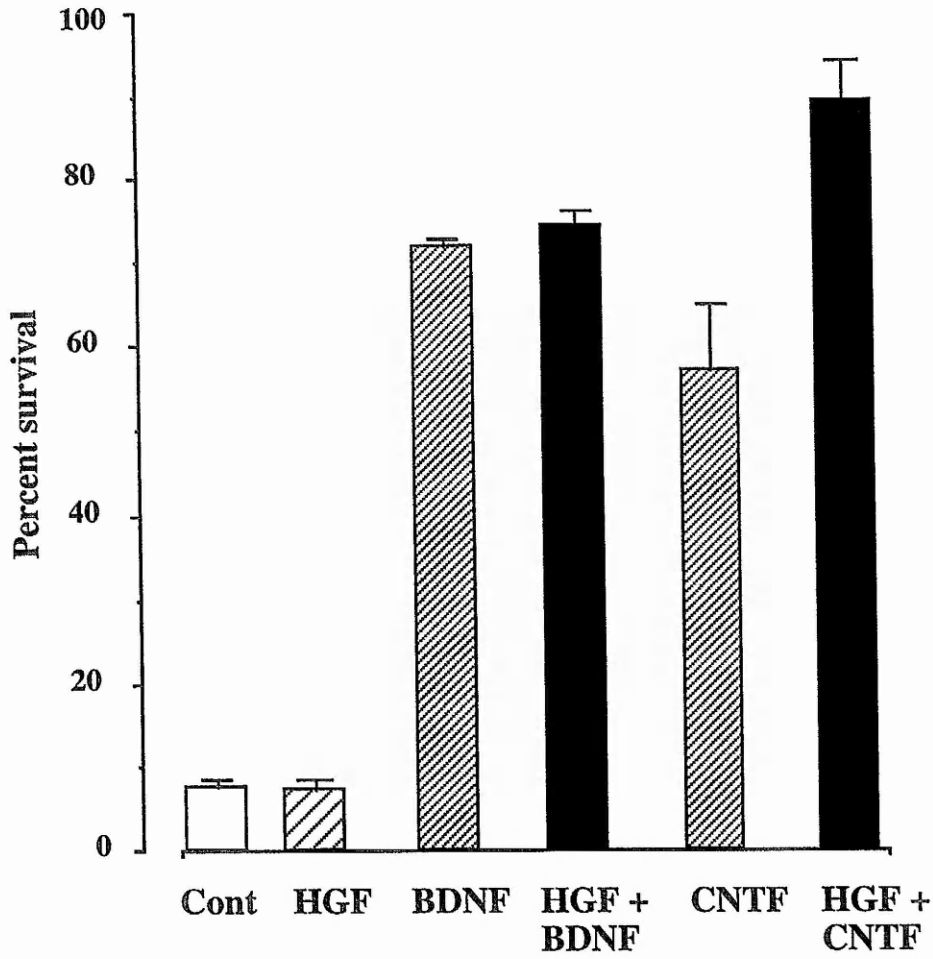


Figure 5.3: Bar chart of the percent survival of E10 TMN neurons incubated for 48 hours in control cultures, cultures containing HGF, BDNF or CNTF alone or in combination (all at 10 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Two additional separate experiments gave very similar results.

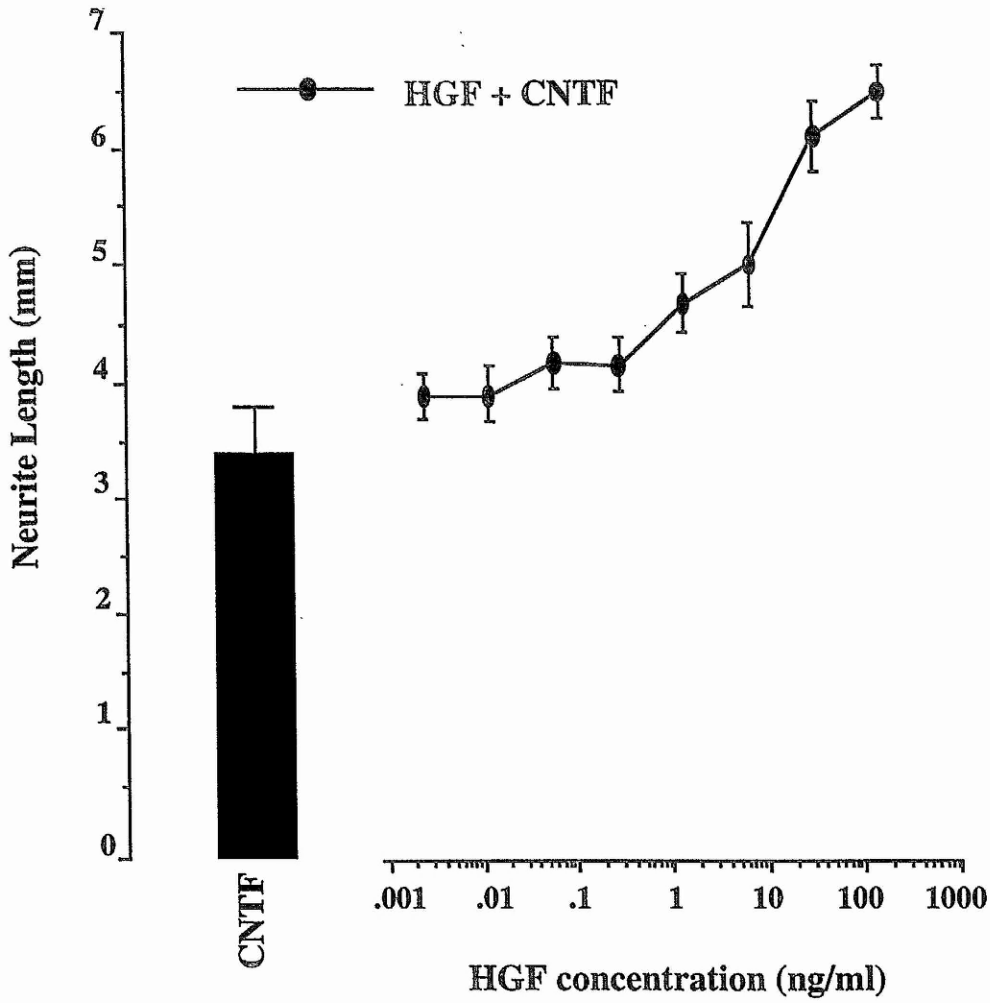


Figure 5.4: Dose responses of the effect of HGF and CNTF on the survival of E10 ciliary ganglion neurons after 48 hours in culture. The graph shows the length (mm) of the neurite arbors of neurons in cultures containing CNTF alone (50 ng/ml) and cultures containing HGF over a range of concentrations with CNTF (50 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Three additional separate experiments gave very similar results.

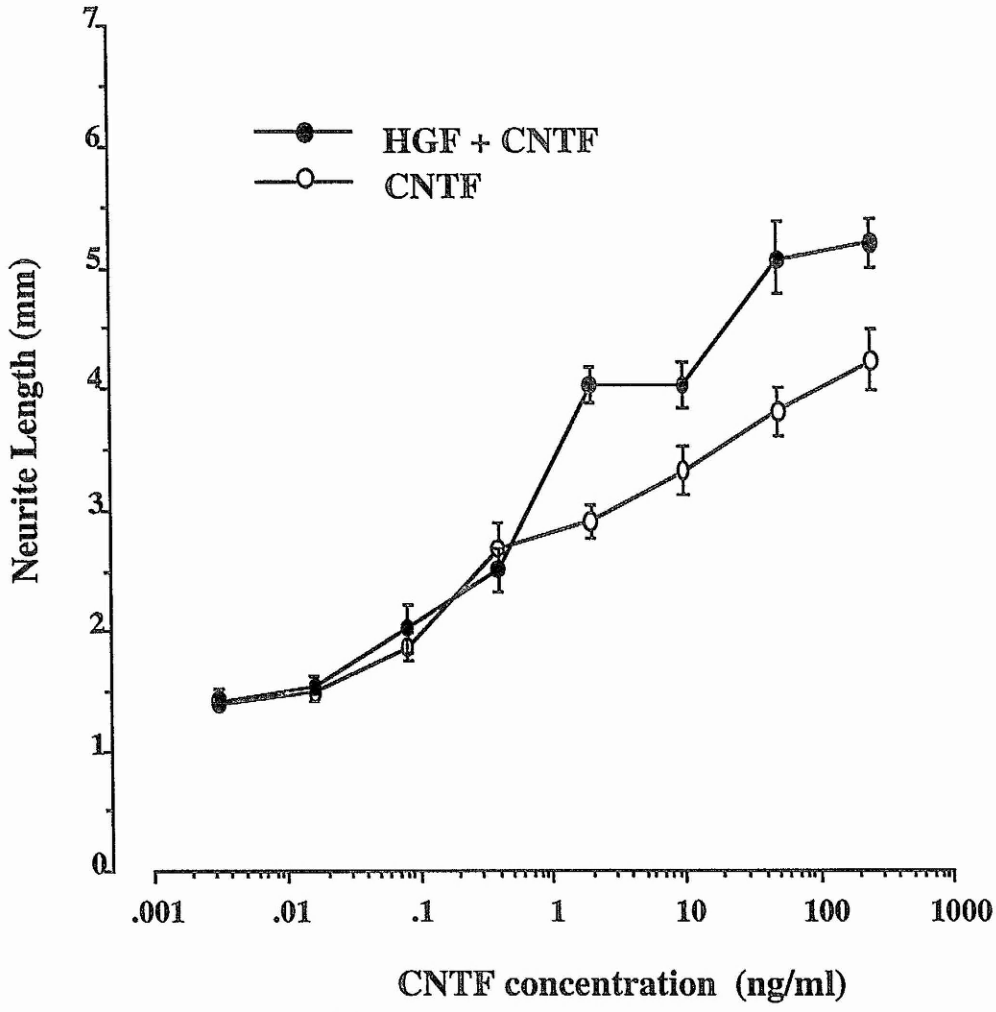


Figure 5.5: Dose responses showing the effect of HGF and CNTF on the growth of E10 ciliary ganglion neurons after 48 hours in culture. The graph shows the length (mm) of the neurite arbors of neurons in culture containing CNTF over a range of concentrations with or without HGF (2 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Three additional separate experiments gave very similar results.

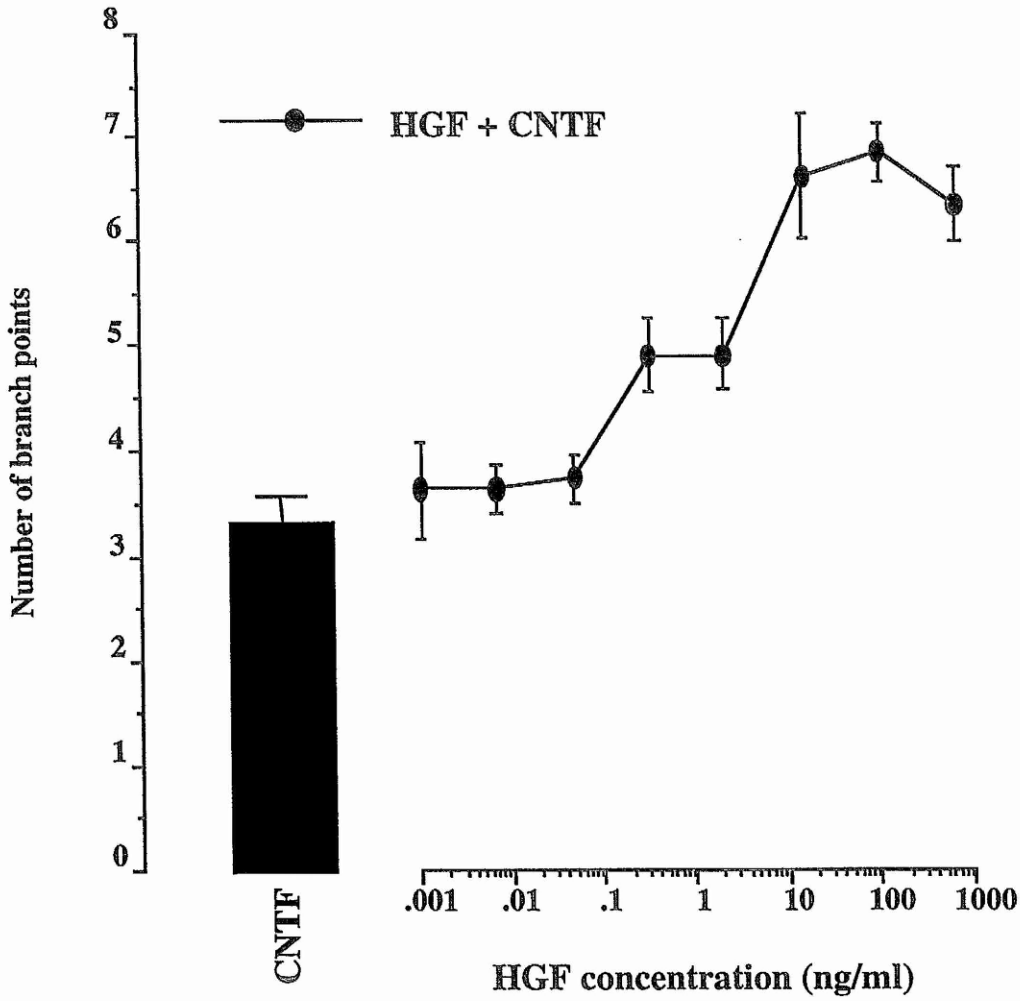


Figure 5.6: Dose responses showing the effect of HGF and CNTF on the branching of E10 ciliary ganglion neurons after 48 hours in culture. The graph shows the number of branch points in neurite arbors of neurons in culture containing CNTF alone (50 ng/ml) and cultures containing HGF over a range of concentrations with CNTF (50 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown . Three additional separate experiments gave very similar results.

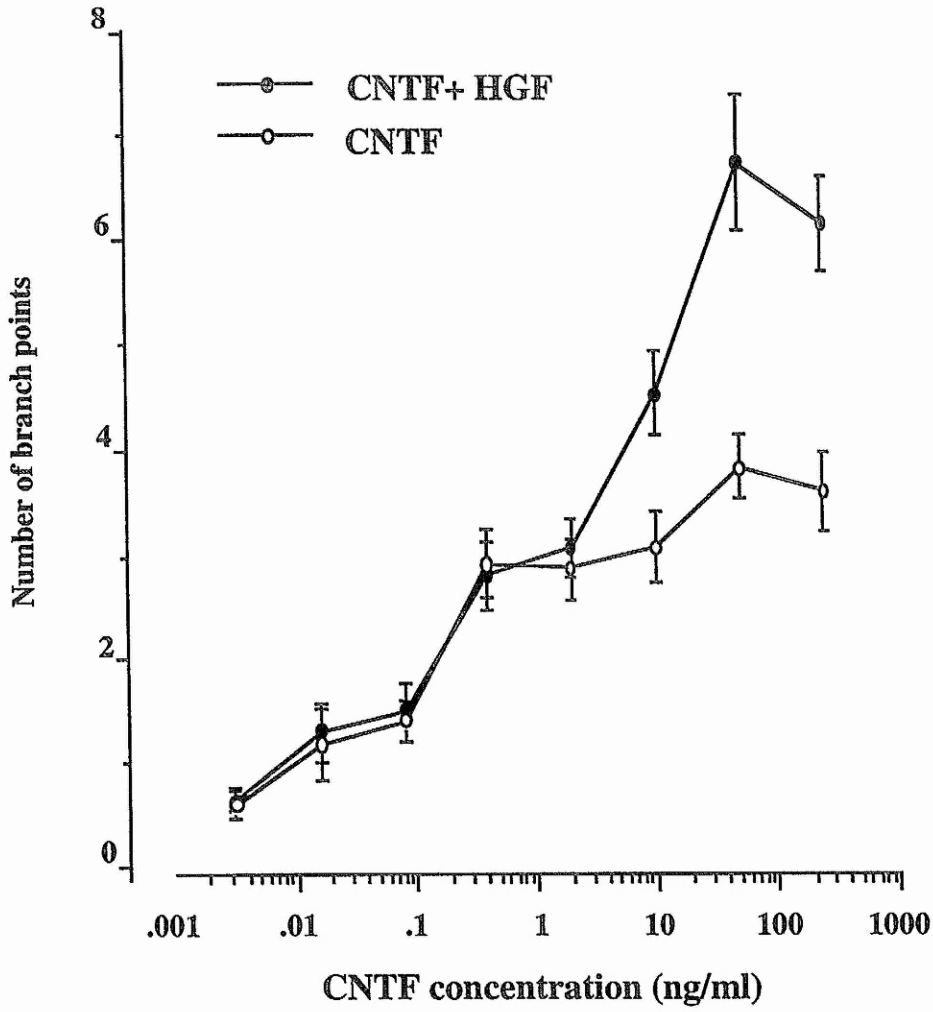


Figure 5.7: Dose responses showing the effect of HGF and CNTF on the branching of E10 ciliary ganglion neurons after 48 hours in culture The graph shows the number of branch points in the neurite arbors of neurons in cultures containing CNTF over a range of concentrations with or without HGF (2 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Three additional separate experiments gave very similar results.

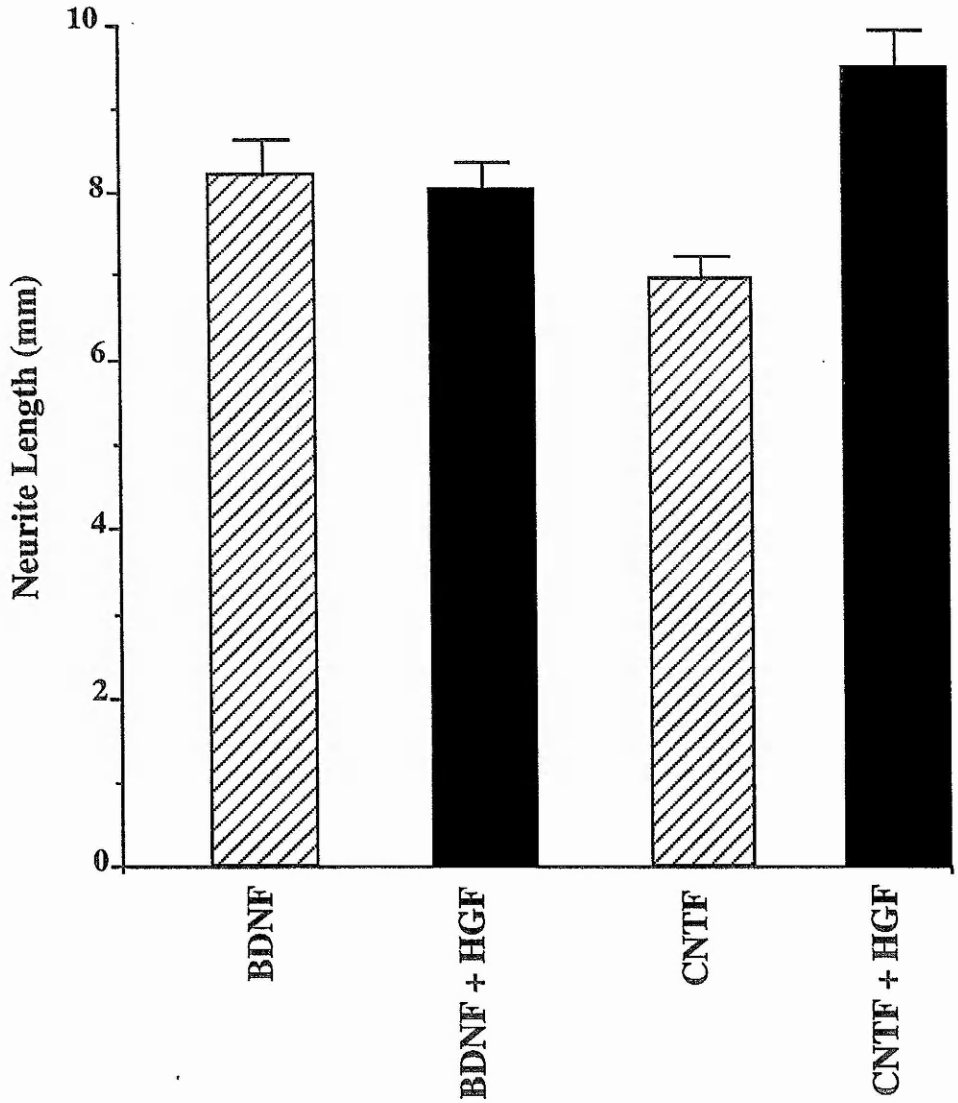


Figure 5.8: Bar chart of the total length of neurite arbors of E10 TMN neurons incubated for 48 hours in control cultures, cultures containing either HGF, BDNF or CNTF alone or in combination (all at 10 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Two additional separate experiments gave very similar results.

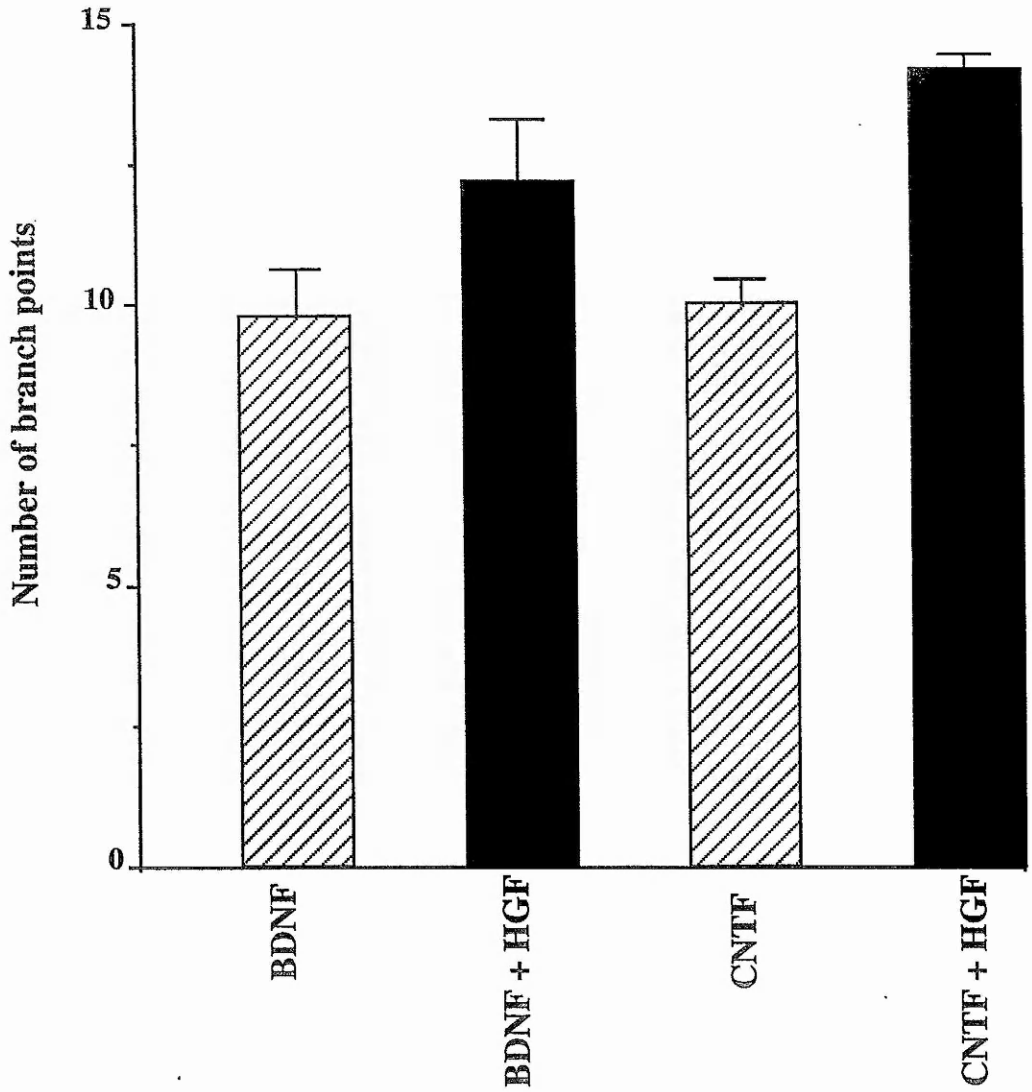


Figure 5.9: Bar chart of the of branch points of E10 TMN neurons incubated for 48 hours in cultures containing either BDNF or CNTF alone or in combination with HGF (all at 10 ng/ml). The mean ± standard error of the mean (n = 3 for each experimental condition) from a typical experiment are shown. Two additional separate experiments gave very similar results.

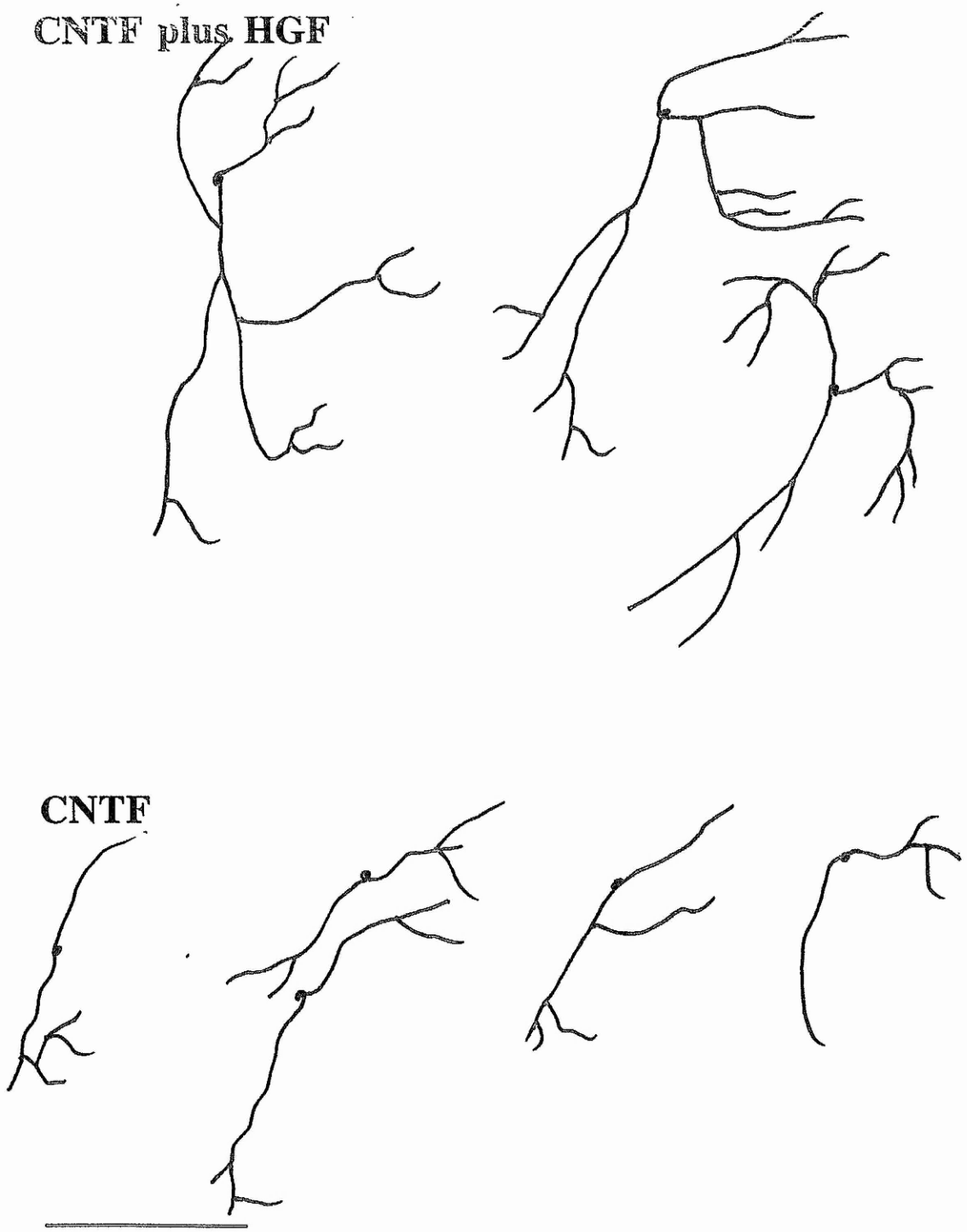


Figure 5.10: Camera lucida drawings of the typical appearance of E10 ciliary ganglion neurons grown for 48 hours with CNTF (50ng/ml) plus HGF (50 ng/ml) or CNTF (50 ng/ml) alone. (Scale Bar = 2mm).

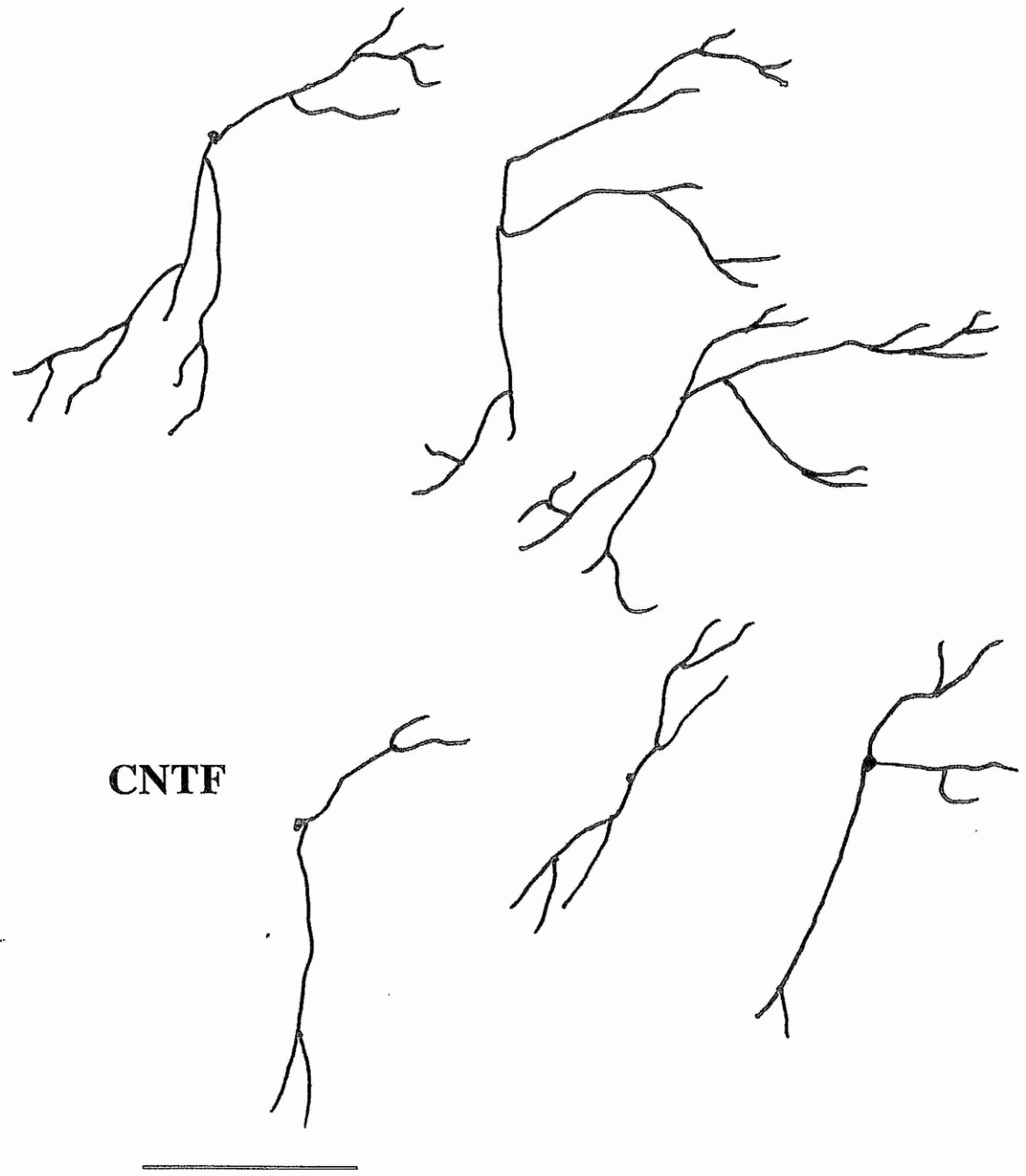


Figure 5.11: Camera lucida drawings of the typical appearance of E10 TMN neurons grown for 48 hours with CNTF (50ng/ml) plus HGF (50 ng/ml) or CNTF (50 ng/ml) alone. (Scale Bar = 2mm).

5.3 DISCUSSION

I have shown that HGF synergises with CNTF in promoting the survival and growth of embryonic parasympathetic and proprioceptive neurons. Although HGF had no effect on the survival of these neurons alone, it consistently promoted a statistically significant elevation in the number of neurons surviving with saturating concentrations of CNTF, and significantly increased the length and branching of the neurite arbors of these neurons in the presence of CNTF. This synergism between HGF and CNTF was due to direct effects of both factors acting on the same neurons because the neurons in these cultures were grown at very low density and there were virtually no other cell types present to mediate an indirect effect.

It has previously been reported that HGF synergises with CNTF in promoting the survival of spinal motoneurons (Wong et al., 1997). However, unlike parasympathetic and proprioceptive neurons which do not survive at all with HGF on its own, a subset of motoneurons does survive with HGF alone, and the number of motoneurons in cultures containing HGF plus CNTF is the sum of the number of motoneurons in cultures containing HGF alone and CNTF alone (Wong et al., 1997). Thus, these data do not necessarily indicate that HGF and CNTF act synergistically on the same motoneurons to enhance their survival, but that HGF and CNTF promote the survival of separate subsets of spinal motoneurons in culture. Although the level of acetyl cholinesterase in motoneuron cultures containing CNTF and NGF was greater than the sum of the levels in cultures containing either factor alone, one cannot exclude the possibility that this apparent synergism between CNTF and HGF in regulating acetyl cholinesterase expression was not mediated indirectly via other cell types which were evident in these cultures (Wong et al., 1997).

Compelling evidence for synergism between HGF and NGF has been obtained from the *in vitro* studies on developing DRG and sympathetic neurons outlined in Chapters 3 and 4. Although HGF does not promote the survival of early DRG neurons

on its own, it enhances the survival of neurons grown with NGF but not those grown in the presence of either BDNF or NT3. HGF also specifically enhances neurite outgrowth from early DRG neurons grown with NGF but not from those grown with other neurotrophins and enhances the growth but not the survival of sympathetic neurons grown with NGF.

Although these studies indicate that HGF can enhance the survival and/or growth of neurons that require NGF for survival, it is not known whether these effects of HGF are specifically dependent on concomitant NGF signalling or whether HGF would have the same effect on this subset of neurons if their survival were promoted by other neurotrophic factors. Because DRG consist of largely separate subsets of NGF, BDNF and NT3-dependent neurons and the NGF-dependent neurons do not uniquely respond to another neurotrophic factor, it has not been possible to address this important question with this subset of neurons. However, in the experiments reported in this Chapter it has been possible to investigate the effect of HGF on a population of neurons that is supported by either of two neurotrophic factors. The majority of TMN neurons survive in culture with either BDNF or CNTF and the combination of both factors does not significantly increase neuronal survival, indicating that the same neurons are supported by either factor (Davies et al., 1986a, 1986b; Allsopp et al., 1995; Davey & Davies, 1998). The demonstration that HGF has no survival promoting effect on these neurons alone but that it enhances the survival and growth of these neurons in the presence of CNTF but not BDNF clearly shows that response of these neurons to HGF depends on the concomitant activation of particular signalling pathways (in this case those activated by CNTF), not just any signalling pathway that promotes survival.

HGF binds to the Met receptor tyrosine kinase resulting in the activation of several signalling pathways including; STAT, JNK kinase, Src tyrosine kinase, Ras/MAP kinase, PI-3 kinase and PLC-γ (Ponzetto et al., 1994; Kochhar and Iyer,

1996; Nguyen et al., 1997; Rodrigues et al., 1997; Boccaccio et al., 1998). CNTF binds to an oligomeric receptor complex comprised of two transmembrane glycoproteins, gp130 and LIFRβ, and a GPI-linked protein, CNTFRα (Davis et al., 1993; Stahl and Yancopoulos, 1994). The activation of this receptor results in STAT, Ras/MAP kinase, PI-3 kinase and PLC-γ activation (Boulton et al., 1994; Stahl et al., 1994; Frank and Greenberg, 1996). Thus, HGF and CNTF have several downstream effectors in common in responsive cells. It will be interesting in future work to ascertain if convergence of HGF and CNTF signalling at one or more of these downstream effectors could explain the synergy between HGF and CNTF.

Chapter 6
Bcl-2 influences axonal growth rate in embryonic neurons

6.1 Introduction

In previous chapters I described a number of extrinsic influences on axonal growth. In this chapter I report the results of studies that implicate Bcl-2, a widely expressed cytoplasmic protein, in influencing axonal growth. Although Bcl-2 is known to play a role in regulating neuronal survival in the course of studying early sensory neurons from Bcl-2 null mutant mouse embryos in vitro, I observed that neurons from Bcl-2 deficient embryos possessed shorter axons than neurons from their wild-type litter mates. I therefore carried out a detailed comparative study of axonal growth rates in low density dissociated neuronal cultures established from Bcl-2 deficient and wild-type embryos.

Because the development of the mouse trigeminal ganglion has been extremely well characterised, I decided to focus on these neurons. During embryonic development the majority of neurons within the trigeminal ganglion differentiate from proliferating progenitor cells between E9.5 and E13.5. The first axons emerge from the trigeminal ganglion at E9.5 and reach their peripheral targets by E11. The latest axons arrive at their targets shortly before E15. The number of neurons within the ganglion peaks between E12 and E14 and thereafter decreases by 50% to reach a stable number by birth as a result of neuronal apoptosis that peaks at E14. The majority of trigeminal neurons require an adequate supply of target field derived NGF to survive this period of cell death (Davies & Lumsden, 1984, 1986: Davies, 1987a, Pinon et al., 1996). Early trigeminal neurons do not require neurotrophic factor support during the period that their axons are growing towards their peripheral targets. Once the axons of early trigeminal neurons have contacted their targets these neurons become dependent on neurotrophic factors for

survival (Buchman & Davies, 1993). In vitro studies have shown that most early trigeminal neurons are dependent upon BDNF or NT-3 for survival and few respond to NGF. However, between E11 and E12 many trigeminal neurons lose the ability to respond to BDNF and acquire responsiveness to NGF. The ability of NT-3 to promote the in vitro survival of trigeminal neurons is gradually lost between E11 and E14 (Buchman & Davies, 1993; Paul & Davies, 1995). The acquisition of NGF dependence is accompanied by marked increases in the expression of transcripts encoding the NGF receptors, p75 and trkA (Wyatt & Davies, 1993).

E11 and E12 are ideal ages at which to study the role of Bcl-2 in regulating process outgrowth from cultured trigeminal neurons for two reasons. First, at these ages a large number of trigeminal neurons are sending axons to their peripheral and central targets in vivo, hence effects of Bcl-2 on neurite outgrowth found at these ages are likely to be of relevence to the process of target field innervation. Second, at E11 and E12 trigeminal neurons respond to BDNF, NT-3 and NGF, thus allowing the role of Bcl-2 in mediating neurite outgrowth in response to all three neurotrophins to be ascertained.

Careful analysis of process outgrowth from E11 and E12 trigeminal neurons obtained from wild type, heterozygous and Bcl-2 deficient mice has shown that Bcl-2 plays a role in the process of neurite outgrowth in response to BDNF, NT-3 and NGF. Importantly, I found that the lack of Bcl-2 did not significantly reduce the survival of E11 and E12 trigeminal neurons grown with any of the neurotrophins. This strongly suggests that the effect of Bcl-2 on axonal growth rate is not a consequence of its well documented role in preventing apoptosis.

6.2 Results

E11 and E12 embryos were obtained from overnight matings of mice that were heterozygous for a Bcl-2 null mutation (Nakayama et al., 1993; Nakayama et al., 1994). Separate low density dissociated cultures of trigeminal ganglion neurons, containing either 5ng/ml NGF, NT-3 or BDNF, were established from each embryo in each E11 and E12 litter. Some neurons are generated from progenitor cells in dissociated cultures of early trigeminal ganglia and therefore start extending axons at different times in culture (Paul & Davies, 1995). Also, Bcl-2 deficient neurons do not survive as well as wild type neurons when cultured during the period of developmental neuronal death (Pinon et al., 1997). Therefore, to eliminate the possibility that the lack of Bcl-2 may alter axonal outgrowth as a secondary consequence of altering neuronal differentiation or survival, individual neurons were identified at the outset of each experiment (after 6 hours in culture) and serial drawings were made of the axons of these identified neurons at regular intervals over a 48 hour culture period. Axon lengths were only calculated from drawings of neurons that survived the whole 48 hour culture period. Tissue from each embryo was saved at the beginning of each experiment for subsequent genotype analysis by PCR. To eliminate the potential for observer bias, drawings were made of axons, and the length of these axons were calculated, without knowing the genotypes of the neurons in the individual cultures. Genotypes were only assigned to cultures after all calculations of neurite length had been made.

Analysis of E11 cultures showed that neurons from Bcl-2 deficient embryos extended their axons at a slower rate than those from either Bcl-2 heterozygous or wild-type litter mates. After 48 hrs incubation, E11 Bcl-2-deficient trigeminal neurons cultured in NGF, BDNF and NT-3 were between 44 and 60% shorter than wild-type E11 neurons (figures 6.1, 6.2 and 6.3). The difference in axonal length between the neurons of Bcl-2 deficient and wild-type embryos were statistically significant at 12, 24, and 48 hrs incubation ($p<0.0001$ in all casesusing t tests). The difference in axonal outgrowth between wild-type and Bcl-2 deficient neurons

was most marked after 48 hrs in culture. Neurons from wild-type and Bcl-2 heterozygous embryos grew at similar rates, suggesting that at E11 the effect of Bcl-2 on promoting axonal growth was independent of gene dosage. Because only neurons that survived throughout the 48 hr period of study were included in the analysis, differences in axonal growth rate between Bcl-2-deficient and wild-type embryos were not due differences in survival. Moreover, there were no significant differences in the overall percentage survival of E11 Bcl-2-deficient and wild-type neurons in these cohorts after 48 hrs incubation with NGF, BDNF and NT-3 ($p>0.05$ in all cases, using t tests). In BDNF supplemented cultures, 55.1 ± 4.4% of neurons survived the 48 hour culture period, whereas 54.1 ± 4.4% Bcl-2 deficient neurons survived. In NT-3 supplemented cultures, E11 neurons of wild-type mice exhibited 55.8 ± 2.9% survival, compared to Bcl-2 deficient neurons which exhibited 49 ± 1.6% survival. In NGF supplemented E11 cultures, 57 ± 5.1% wild type neurons survived, compared to 51 ± 4.3% Bcl-2 deficient neurons.

Analysis of E12 cultures showed that neurons from Bcl-2 deficient embryos also extended their axons at a slower rate than those from either Bcl-2 heterozygous or wild-type litter mates. After 48 hrs incubation, E12 Bcl-2-deficient neurons cultured in NGF, BDNF and NT-3 were between 35% and 50% shorter than wild-type E12 trigeminal neurons (figures 6.4, 6.5 and 6.6). The difference in axonal length between the neurons of Bcl-2 deficient and wild-type embryos was statistically significant at 12, 24, and 48 hrs incubation ($p<0.0001$ in all cases, using t tests). As at E11, the difference in axonal outgrowth between E12 wild-type and Bcl-2 deficient neurons was most marked after 48 hrs in culture and neurons from E12 Bcl-2 heterozygous and wild-type embryos grew at similar rates. In BDNF supplemented cultures, 59.8 ± 3.3% of E12 wild type trigeminal neurons survived for 48 hours compared to 51 ± 3.9% Bcl-2 deficient neurons. In NT-3 supplemented cultures, the survival of wild type and Bcl-2 deficient trigeminal neurons was 57 ± 2.5% and 48 ± 3.0%, respectively. In NGF supplemented cultures, E12 neurons of wild-type mice exhibited 59 ± 2.4% survival, compared to

Bcl-2 deficient neurons which exhibited 56 ± 2.6% survival. Since, there were no significant differences in the overall percentage survival of E12 Bcl-2-deficient and wild-type neurons in identified cohorts after 48 hrs incubation under each experimental condition ($p>0.05$ in all cases, using t tests), the effect of Bcl-2 on neurite outgrowth cannot be secondary to effects of Bcl-2 on promoting survival. The typical appearances of Bcl-2 deficient and wild-type E11 and E12 trigeminal neurons grown for 48 hrs with NGF are shown in Figures 6.7 and 6.8.

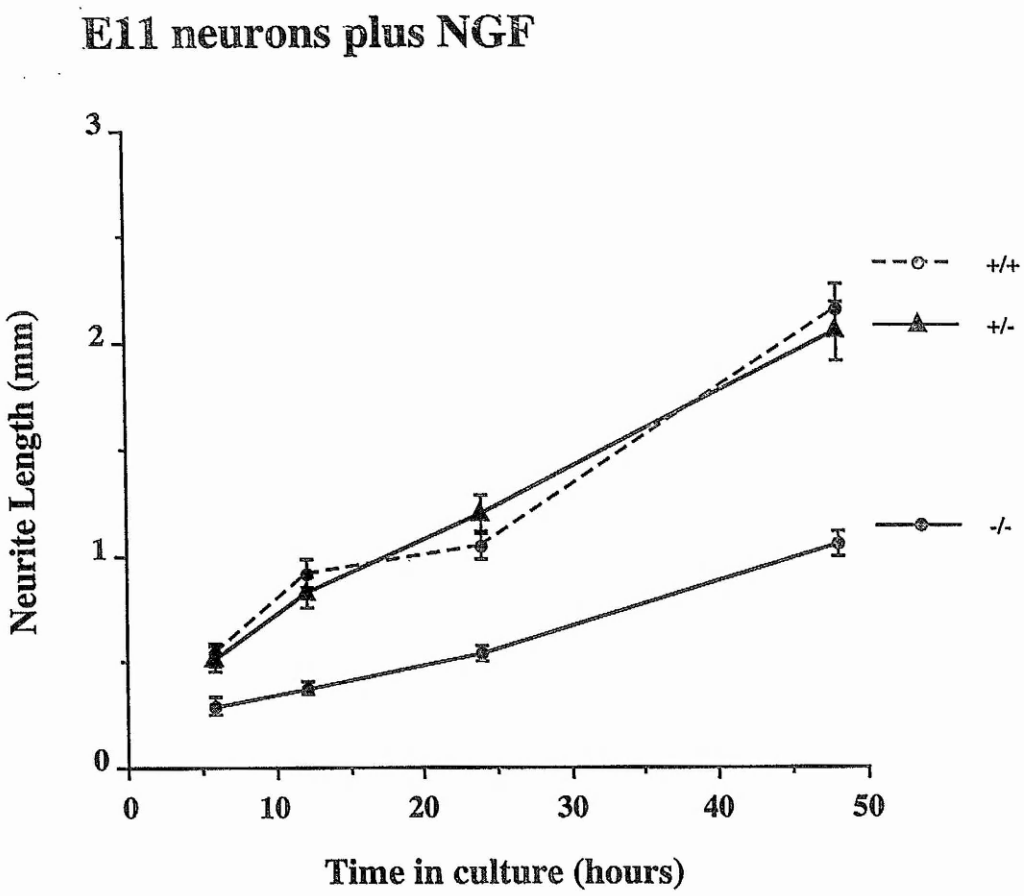


Figure 6.1: Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild-type embryos, in E11 cultures in NGF (5ng/ml). The means and standard errors are shown of serial measurements made on between 150-and-100 neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=6, Bcl-2+/- n=9, Bcl-2-/- n=7.

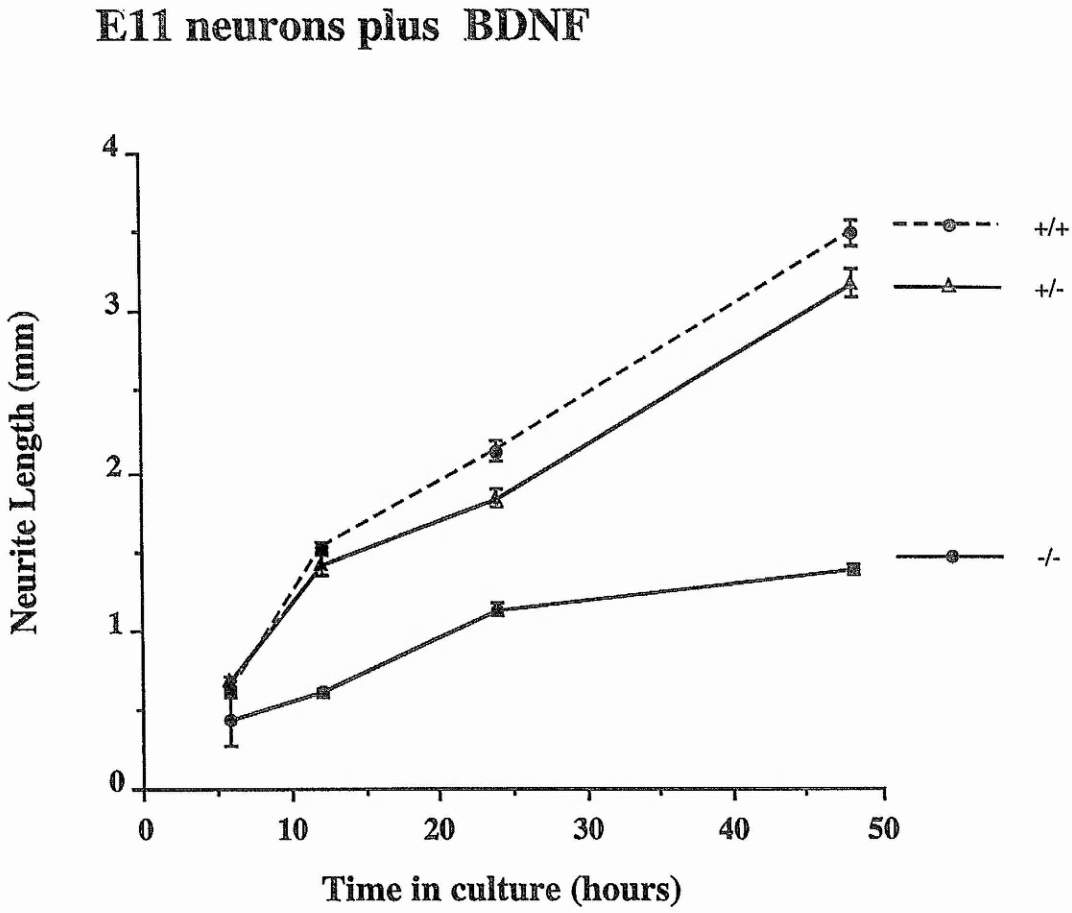


Figure 6.2: Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild-type embryos, in E11 cultures in BDNF (5ng/ml). The means and standard errors are shown of serial measurements made on between 150-and-100neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=6, Bcl-2+/- n=9, Bcl-2-/- n=7.

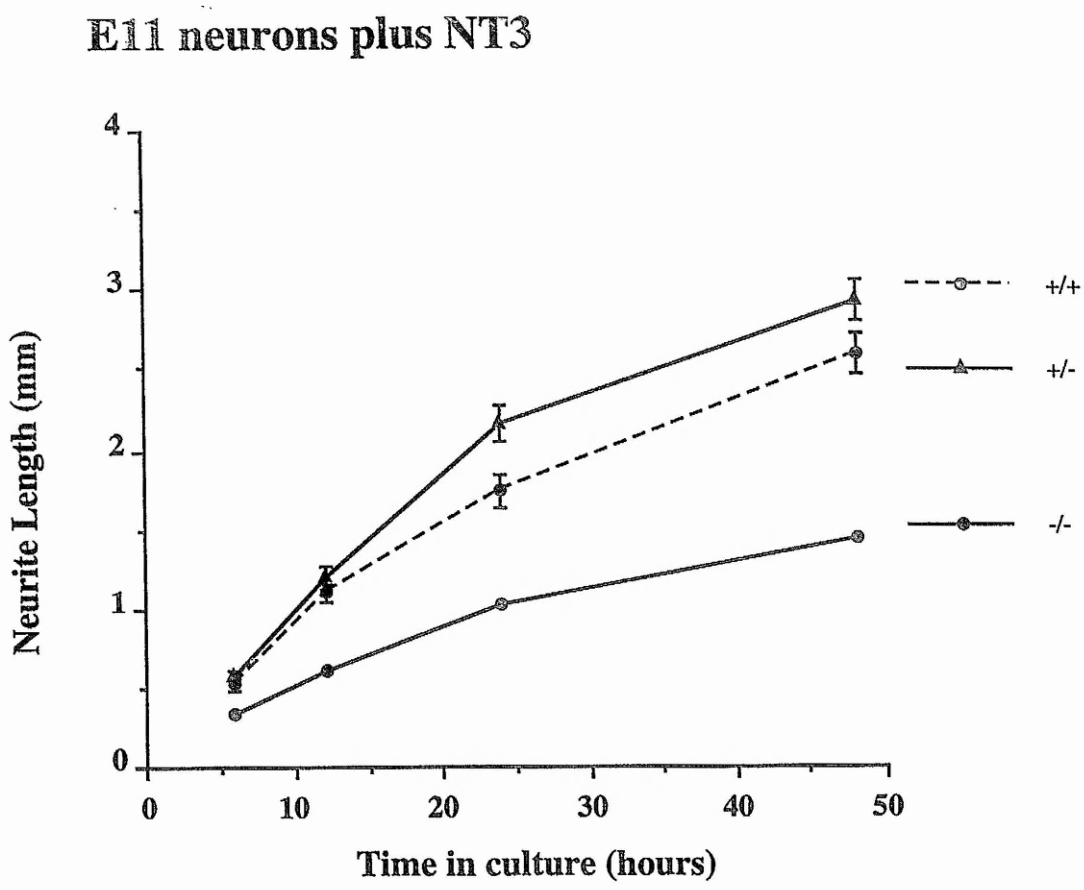


Figure 6.3 Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild-type embryos, in E11 cultures in NT-3 (5ng/ml). The means and standard errors are shown of serial measurements made on between 100-and-150neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=6, Bcl-2+/- n=9, Bcl-2-/- n=7.

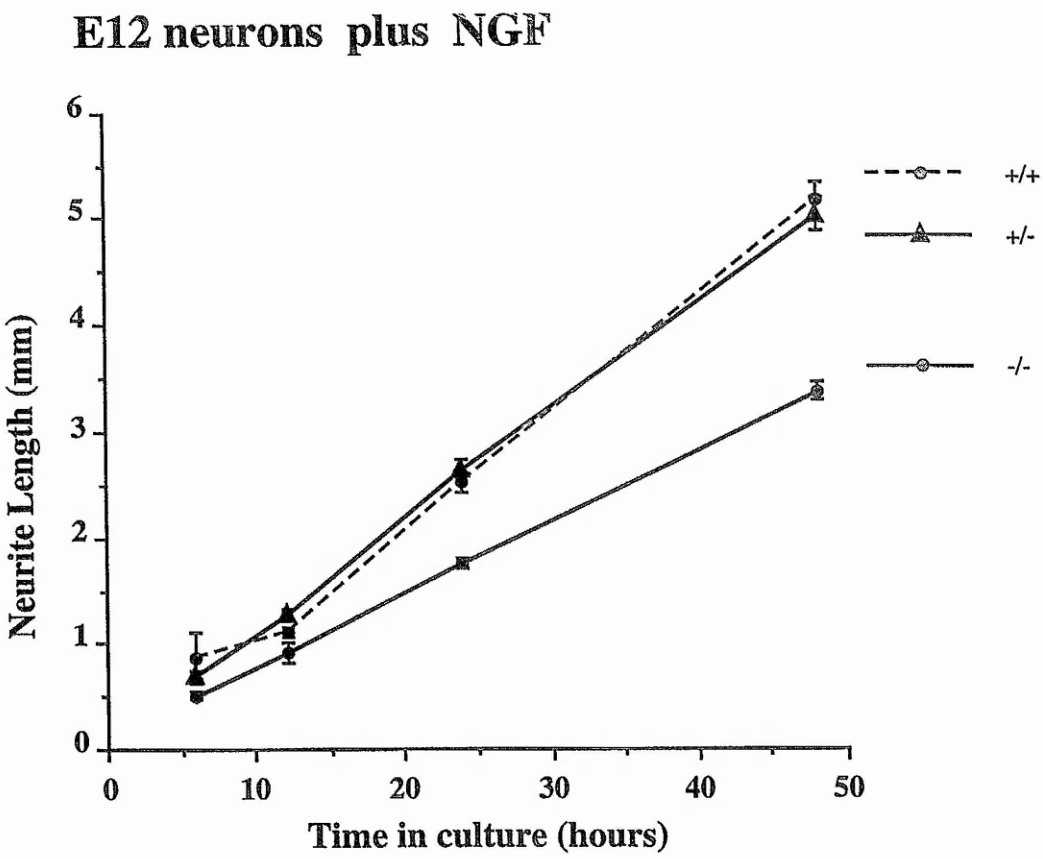


Figure 6.4: Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from Bcl-2-deficient, heterozygous and wild-type embryos, in E12 cultures in NGF (5ng/ml). The means and standard errors are shown of serial measurements made on between 150-and-100 neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=5, Bcl-2+/- n=7, Bcl-2-/- n=5.

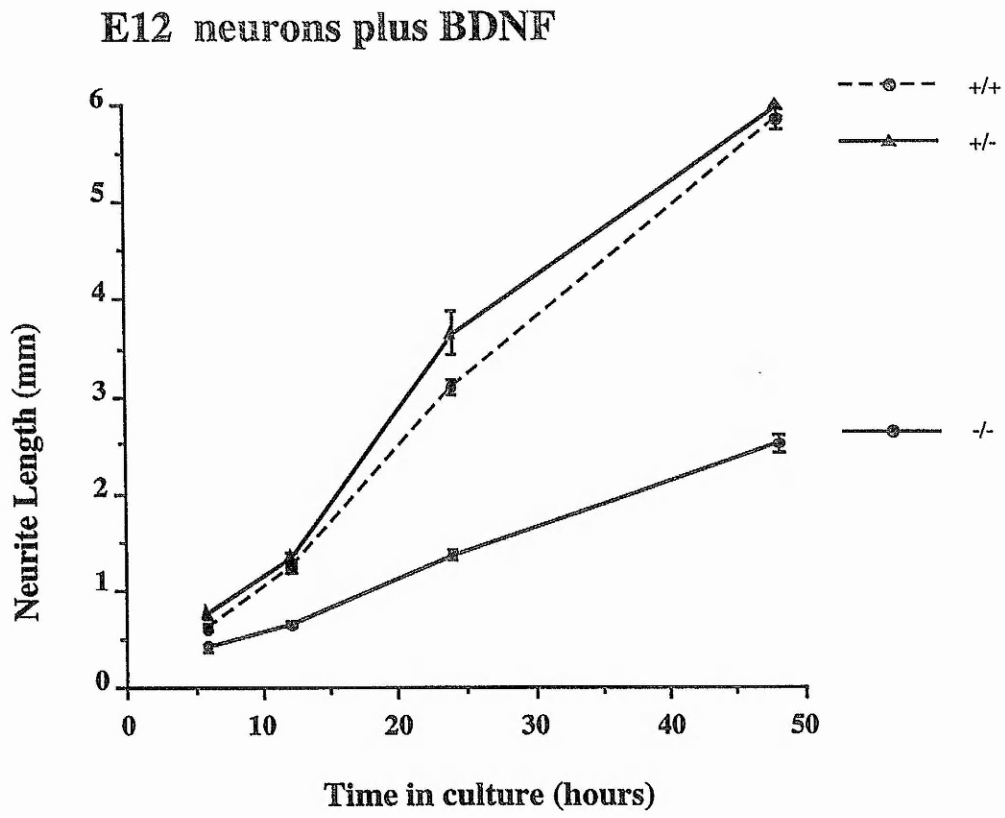


Figure 6.5: Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from-2-deficient, heterozygous and wild-type embryos, in E12 cultures in BDNF (5ng/ml). The means and standard errors are shown of serial measurements made on between 150-and-100 neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=5, Bcl-2+/- n=7, Bcl-2-/- n=5.

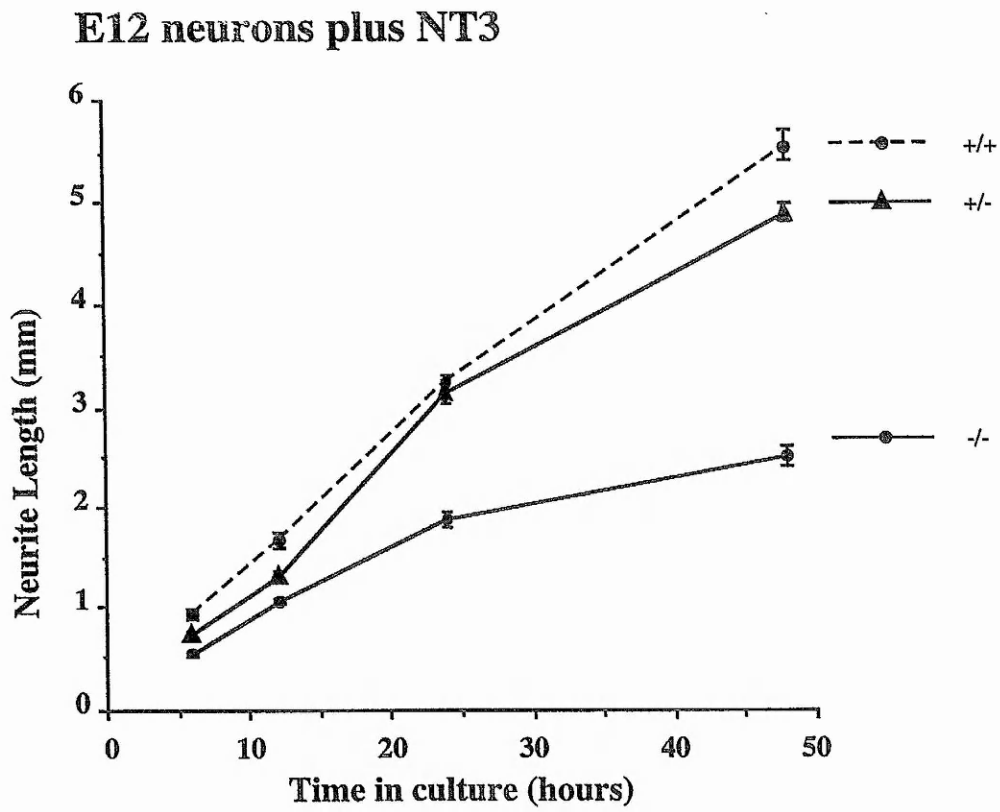


Figure 6.6: Line graphs showing the results of neuron cohort experiment comparing axonal growth rates of trigeminal neurons from-2-deficient, heterozygous and wild-type embryos, in E12 cultures in NT-3 (5ng/ml). The means and standard errors are shown of serial measurements made on between 150-and-100 neurons of each genotype, compiled from cultures set up from the embryos of three separate litters, Bcl-2+/+ n=5, Bcl-2+/- n=7, Bcl-2-/- n=5.

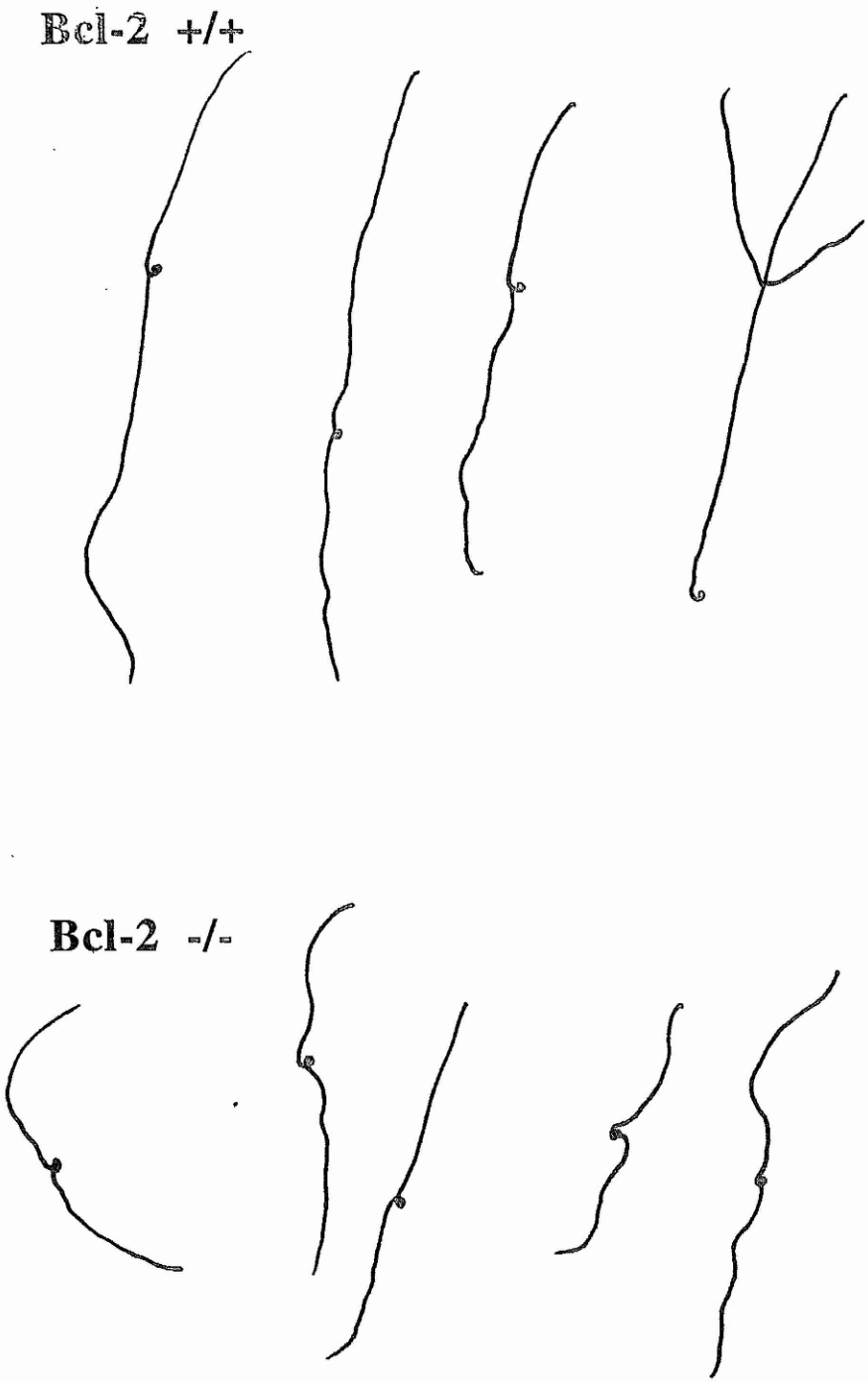


Figure 6.7: Camera lucida drawings of the typical appearance of E11 trigeminal neurons from Bcl-2 deficient and wild-type embryos grown for 48 hours with NGF (5ng/ml). (Scale Bar = 2mm).

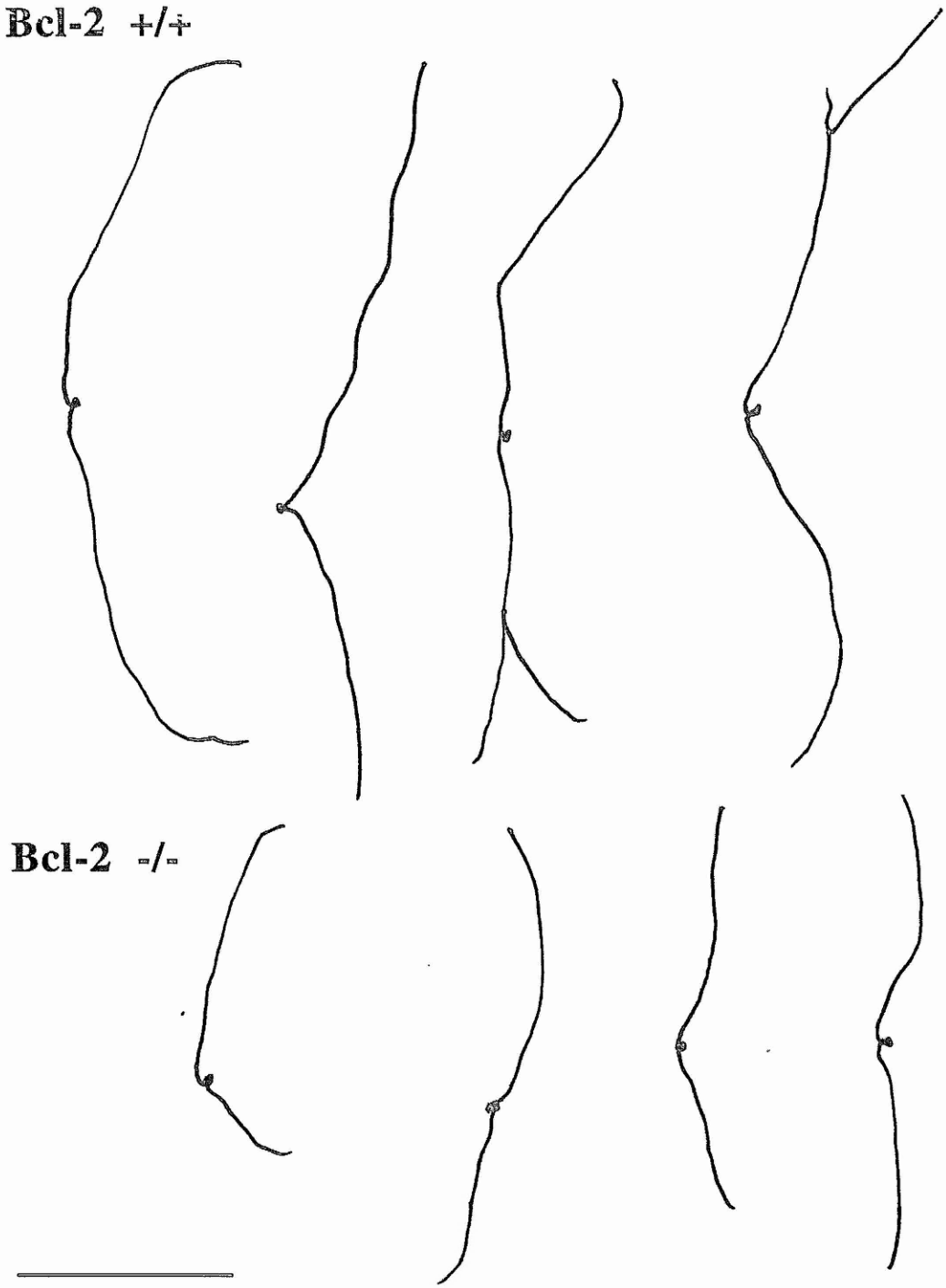


Figure 6.8: Camera lucida drawings of the typical appearance of E12 trigeminal neurons from Bcl-2 deficient and wild-type embryos grown for 48 hours with NGF (5ng/ml). (Scale Bar = 2mm).

6.3 Discussion.

In this study I have obtained clear evidence for a novel role of Bcl-2 during the early stages of neuronal development that is distinct from its well characterized anti-apoptotic function. I have demonstrated that sensory neurons from trigeminal ganglia of E11 and E12 Bcl-2 deficient mouse embryos, cultured with either NT-3, NGF or BDNF, extend axons more slowly in vitro than neurons from wild-type embryos of the same age (Hilton et al., 1997). These results suggest that endogenous Bcl-2 expression influences axonal growth rate in early sensory neurons in culture. Recent evidence supporting the theory that members of the Bcl-2 family can influence axonal growth has been shown by the demonstration that the level of Bcl-2 expression in mouse trigeminal retinal ganglion cells (RGCs) influences the ability of these cells axons to regenerate into co-cultured tectal explants (Chen et al., 1997). At early fetal stages, RGCs express high levels of Bcl-2 and are able to extend axons into co-cultured tectal tissue of the same age, whereas late fetal RGCs express low levels of Bcl-2 and are not able to grow axons into tectal tissue. The regenerative ability of early fetal RGCs is substantially reduced in retinal explants obtained from Bcl-2 deficient embryos, and late fetal and adult RGCs from transgenic mice over expressing Bcl-2 in neurons retain the ability to regenerate into tectal tissue. Likewise, optic nerve axons in Bcl-2 overexpressing transgenic mice show enhanced regenerative capacity following lesion in vivo (Chen et al., 1997). Although these observations provide evidence that Bcl-2 can promote axonal regeneration in the central nervous system, their significance with regards to the role of endogenous Bcl-2 in the regulating the growth of axons to their target during development is uncertain. Furthermore, because the numbers of regenerating RGCs, rather than the growth rates of individual axons, were measured in this study, it is unclear whether the level of Bcl-2 expression affects the growth of regenerating axons or simply affects the proportion of neurons that are able to regenerate following axotomy.

Several previous studies of tumor cell lines have also raised the possibility that Bcl-2 might play a role in neuronal differentiation. First, Bcl-2 overexpression in a neural crest-derived cell line enhances the outgrowth of neurite-like processes and increases the expression of neuron-specific enolase (Zhang et al., 1996). Second, midbrain-derived dopaminergic cell lines stably expressing Bcl-2 extend longer neurites than control transfected cells but do not show increased expression of many neuron-specific proteins (Oh et al., 1996). Finally, Bcl-2 enhances the differentiation of PC12 cells into sympathetic-like neurons when these cells are grown without serum, but not when grown with serum (Batistatou et al., 1993). The physiological and developmental significance of overexpressing Bcl-2 in tumor cell lines is, however, difficult to interpret.

Recently Bcl-2 has been shown to accelerate the maturation of early sensory neurons (Middleton et al., 1998). Shortly after differentiating from progenitor cells, sensory neurons undergo a distinct morphological change; initially they possess small, spindle-shaped, phase-dark cell bodies that later become large, spherical and phase bright. In vitro studies have shown that E11 and E12 trigeminal sensory neurons from Bcl-2 deficient embryos undergo these morphological change at a slower rate than neurons from wild type embryos. The delay in neuronal maturation is not attributable to the well characterized role of Bcl-2 in preventing apoptosis, as Bcl-2 deficient neurons were shown to survive as well as wild-type neurons at these ages. Trigeminal ganglia in early Bcl-2 deficient embryos contain a significantly smaller number of the more mature type of neurons than those in wild type embryos, yet the total number of neurons in the trigeminal ganglia of Bcl-2 deficient and wild-type embryos is similar. The absence of Bcl-2 does not appear to cause a uniform delay in the developmental program of sensory neurons, as the time course and level of expression of trkA and p75 mRNAs are virtually identical in embryos of both Bcl-2 deficient and wild-type mice (Middleton et al., 1998). The demonstration that Bcl-2 accelerates the maturation of early sensory neurons could suggest that the observed reduction in axonal growth rates in Bcl-2 deficient

neurons could be due to a delay in maturation. However, analysis of trigeminal neurons from wild type embryos and heterozygous Bcl-2 embryos has demonstrated that axonal growth is independent of Bcl-2 gene dosage, whereas maturation of immature neurons is not. This argues strongly that the role that Bcl-2 plays in promoting maturation is distinct from the role that it plays in promoting axonal growth.

Recently, the pro-apoptotic Bcl-2 protein family member Bax has also been implicated in playing a role in regulating axonal growth rates (Lentz et al., 1999). The axons of bipolar DRG neurons from Bax deficient embryos are significantly smaller in length than those of bipolar neurons from wild-type embryos. After 72 hrs incubation with NGF, the total length of axons from bipolar Bax deficient neurons is only 65% of that from wild type neurons. In contrast, the length of axons projecting from unipolar Bax deficient neurons cultured with NGF is not reduced compared to unipolar wild type neurons. Bipolar Bax deficient neurons cultured in other neurotrophins also do not appear to exibit shorter axons than wild type neurons cultured with the same neurotrophin (Lentz et al., 1999).

The molecular mechanisms and signal transduction pathways that mediate the effects of Bcl-2 on axonal outgrowth are unknown. However Bcl-2 has been shown to interact with R-ras p23, a member of the Ras family of GTPase proteins (Fernandez-Sarabia & Bischoff, 1993) and is co-immunoprecipitated with the serine/threonine specific protein kinase Raf-1 (Wang et al., 1994). These proteins have been shown to participate as components of growth-factor-mediated signal transduction pathways that regulate neurite outgrowth (Green & Kaplan, 1995). Whether the observed effect of Bcl-2 on the rate of axonal growth in developing sensory neurons is dependent upon these molecules has yet to be examined.

In summary, the data presented in this chapter iare consistent with a novel function for endogenously expressed Bcl-2 in embryonic sensory neurons shortly after they differentiate from progenitor cells and start extending axons to their targets. At this stage of development, Bcl-2 appears to enhance the rate of axonal

growth. This action of Bcl-2 is not related to its well characterised anti-apoptotic role and may provide an explanation for the widespread expression of Bcl-2 in the embryonic nervous system during the earliest stages of neuronal development (Abe et al 1993, Merry et al 1994). Future comparative studies of axonal growth rate in Bcl-2 mutant and wild-type embryos *in vivo* will be crucial in determining the significance of the in vitro observations described in this chapter.

CHAPTER 7

Conclusion

The results presented in this thesis provide evidence for the role of intrinsic and extrinsic factors in regulating the axonal growth rate and survival of developing neural crest and placode derived neurons.

During embryogenesis, the HGF/Met system was shown to be required for the development of mice sensory neurons of the dorsal root ganglia (DRG). Although HGF alone did not promote neurite outgrowth in vitro, it was shown to markedly enhance the outgrowth that occurs in response to NGF, this observation was confirmed using DRG explants. In Vitro, HGF was also shown to enhance the numerous effects of NGF, including the maturation of sensory neuron progenitors, as well as their survival. The synergy with NGF seems to be specific; even though Met expression was observed in populations of DRG neurons that depend on BDNF and NT-3 rather than NGF for survival, HGF did not enhance responses to these neurotrophins. Similar results have been found with cranial sensory neurons from the trigeminal and nodose ganglia, in which effects of BDNF and NT-3 are not enhanced by HGF. Such specificity could be due to differences in signalling between the different neurotrophin receptors.

Similar experiments were done on developing mice sympathetic neurons. Sympathetic ganglia are neural-crest derived structures in which proliferating neuroblasts mature in to postmitotic sympathetic neurons. In early sympathetic ganglia, HGF seems to be produced by the neuroblasts themselves, suggesting that HGF acts in an autocrine or local paracrine mode. Experiments in which the fate of single cells were followed over time in culture demonstrated that endogenously produced HGF enhanced

the survival and differentiation, but not the proliferation, of sympathetic neuroblasts. Consistent with these *in vitro* effects, sympathetic ganglia of met mutant mice showed an approximately twofold increase in apoptotic cells, and a progressive reduction in the total number of cells. As with sensory neurons, HGF was shown to cooperate with NGF to enhance sympathetic neuron axonal outgrowth, and also to increase the numbers of neurites emerging from the cell bodies. In contrast to sensory neurons, however, HGF failed to cooperate with NGF in promoting the survival of postmitotic sympathetic neurons, suggesting that in sympathetic cells, met activates the survival pathways only weakly if at all.

To determine if HGF co-operates with other neurotrophic factors in regulating the survival and growth of other kinds of neurons in the developing peripheral nervous system, I studied the effect of HGF on the parasympathetic neurons of the ciliary ganglion and the proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN), both of which survive and extend neurites in response to CNTF. HGF alone did not promote the survival of either of these kinds of neurons but increased the number of neurons surviving in cultures supplemented with CNTF. HGF also increased the overall length and branching of the neurite arbors of these neurons in the presence of CNTF. These results show that HGF is able to co-operate with CNTF in promoting the survival and growth of developing parasympathetic and proprioceptive neurons. The demonstration that HGF only enhanced the survival and growth of TMN neurons when these neurons were grown with CNTF and not when they were grown with BDNF (which promoted their survival as effectively as CNTF) demonstrates that within the same neurons, the effects of HGF on survival and growth are selectively dependent on which other signalling pathways are concurrently activated.

Finally, I examined the influence of the cytoplasmic protein Bcl-2 on regulating axonal growth rates. previous studies showed that overexpression of Bcl-2 in cultured neurons prevented their death following neurotrophin deprivation. Cultured neurons

expressing antisense Bcl-2 RNA have an attenuated survival response to neurotrophins and neurons of postnatal Bcl-2 deficient mice die more rapidly following NGF deprivation *in vitro* and are present in reduced numbers *in vivo*Serial measurements of axonal length in the same neurons revealed that there were marked differences in axonal growth rate between Bcl-2-deficient and wild type neurons irrespective of whether the neurons were grown with nerve growth factor, brain-derived neurotrophic factor or neurotrophin-3. Because there was no significant difference in the numbers of wild type and Bcl-2-deficient neurons surviving with each neurotrophin at this early stage of development, the effect of Bcl-2 on axonal growth rate was not a consequence of its well-documented role in preventing apoptosis.

References

Abe, D. S, Harada, N., Yamada, K., and Tanaka, R. (1993). Bcl-2 gene is highly expressed during neurogenesis in the central nervous system *Biochem. Biophys. Res. Commun.* 191:915-921.

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. Anat. Rec. *94*: 239-247.

Acheson, A., Conover, J. C., Fandl, J. P., DeChlara, T. M., Russell, M., Thadani, A., Squinto, S.P., Yancopoulos, G. D., and Lindsay, R. M. (1995), A BDNF autocrine loop in adult sensory neurons prevents cell death.Nature 374: 450-453.

Adler, R., Landa, K. B., Manthorpe, M. and Varon, S. (1979). Cholinergic neuronotrophic factors: intraocular distribution of trophic activity for ciliary neurons. Science 204:1434-1436

Albers, K. M., Perrone, T. N., Goodness, T. P., Jones, M. E., Green, M. A. and Davis, B. M. (1996). Cutaneous overexpression of NT-3 increases senosry and sympathetic neurons number and enhances touch dome and hair follicle innervation. *J. Cell Biol.* 134, 487-497.

Albers, K. M., Wright, D. E. and Davis, B. M. (1994). Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. J Neurosci , 3: 1422-1432.

Alderson, R. F., Alterman, A. L., Barde, Y. A., and Lindsay, R. M. (1990). Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron. 5:297-306.

Allsopp, T. E,. Wyatt, S., Paterson, H.F., and Davies, A.M. (1993). The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* 73:295-307.

Allsopp, T. E., Kiselev, S., Wyatt, S., and Davies, A. M. (1995) Role of Bcl-2 expression in the BDNF survival response. *Euro. J* .1995, 7: 1266-1272 .

Andermarcher, E., Surani, M. A., and Gherardi, E. (1996). Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. Dev. Genet. *18*: 254-266.

Anderson, D. J., and Axel, R. (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. Cell *47*: 1079-1090.

Angeletti, P., and Vigneti, E. (1971). Assay of nerve growth factor (NGF) in subcellular fractions of peripheral tissues by micro complement. fixation. Brain Res. 33:601-604

Angeletti, R. H. and Bradshaw, R. A. (1971a). Nerve growth factor from mouse submaxillary gland: amino acid sequence. *Proc. Natl. Acad. Sci. U S A* 68: 2417-2420.

Angeletti, R. H., Bradshaw, R. A., and Wade, R. D. (1971). Subunit structure and amino acid sequence of mouse submaxillary gland nerve growth factor. Biochemistry. 10: 463-469.

Angeletti, R. H., Mercanti, D. and Bradshaw, R. A. (1973). Amino acid sequences of mouse 2.5S nerve growth factor. I. Isolation and characterization of the soluble tryptic and chymotryptic peptides. *Biochemistry* 12: 90-100.

Arakawa, Y., Sendtner, M. and Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. *J Neurosci* 10: 3507-15.

Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985). Cloning the chromosomal breakpoint of t(14;18) humanlymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell. 41: 899-906.

Baloh, R. H., Tansey, M. G., Golden, J. P., Creedon, D. J., Heuckeroth, R. O., Keck, C. L., Zimonjic, D. B., Popescu, N. C., Johnson, E. M. J. and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* 18: 793-802.

Baloh, R. H., Gorodinsky, A., Golden, J. P., Tansey, M. G., Keck, C. L., Popescu, N. C., Johnson, E. M. J., and Milbrandt, J. (1998a). GFRa3 ia an orphan member of the GDNF/neurturin/persephin receptor family. Proc. Natl. Acad. Sci. USA. 95: 5801-5806.

Baloh, R. H., Tansey, M. G., Lampe, P. A., Fahrner, T. J., Enomoto, H., Simburger, K. S., Leitner, M. L., Araki, T., Johnson, E. M. J., and Milbrandt, J.(1998b). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRa3-Ret receptor complex. Neuron 21: 1291-1302.

Bamber, B. A., Masters, B. A., Hoyle, G. W., Brinster, R. L. and Palmiter, R. D. (1994). Leukemia inhibitory factor induces neurotransmitter switching in transgenic mice. *Proc. Natl. Acad. Sci. U S A.* 91: 7839-7843.

Bamji, S. X., Majdan, M., Pozniak, C. D., Belliveau, D. J., Aloyz R, Kohn.J., Causing, C. G., and Miller, F. D. (1998). The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J. Cell. Biol. 140:911-923.

Banner, L. R. and Patterson, P. H. (1994). Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. *Proc.. Natl.. Acad.. Sci.. USA.* 91: 7109-7113.

Barbacid, M. (1994). The Trk family of neurotrophin receptors. J. Neurobiol. 25:1386-1403

Barbacid, M. 1995. Neurotrophic factors and their receptors. *Current Opinion in Cell Biology* 7: 148-155.

Barbin, G., Manthorpe, M. and Varon, S. (1984). Purification of the chick eye ciliary neuronotrophic factor. *J Neurochem* 43: 1468-78.

Barbin, G., Manthorpe, M., and Varon, S. (1984). Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* 43: 1468-1478.

Barde, Y. A., Edgar, D. and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* 1: 549-553.

Barde, Y. A. (1988). What, if anything, is a neurotrophic factor? Trends Neurosci. 11:343-346.

Barde, Y. A. (1989). Trophic factors and neuronal survival. Neuron 6:1525-1534.

Bardelli, A. Maina, F., Gout, I., Fry, M. J., Warefield, M. D., Comoglio, P. M., and Ponzetto, C. (1992). Autophosphorylation promotes complex formation of recombinant hepatocyte growth factor receptor with cytoplasmic effectors containing SH2 domains. Oncogene. 7: 1973-1978.

Bardelli, A., Longati, P., Albero, D., Goruppi, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996). HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. EMBO. J. 15: 6205-6212.

Barinaga, M. 1994. Neurotrophic factors enter the clinic. *Science.* 264: 772-774.

Barker, P. A., Lomen-Hoerth, C., Gensch, E. M., Meakin, S. O., Glass, D. J. and Shooter, E. M. (1993). Tissue-specific alternative splicing generates two isoforms of the *trk*A receptor. *J. Biol. Chem.* 268: 15150-15157.

Barker, P. A. and Shooter, E. M. (1994). Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* 13: 203-215.

Barrett, G. L. and Bartlett, P. F. (1994). The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc. Natl. Acad. Sci. USA* 91: 6501-6505.

Batistatou, A., Merry, D. E., Korsmeyer, S. J., Greene, L. A. (1993) Bcl-2 affects survival but not neuronal differentiation of PC12 cells. *J Neurosci* .13:4422-4428.

Bazan, J. F. (1991). Neuropoietic cytokines in the hematopoietic fold. *Neuron* 7: 197-208.

Benedetti, M., Levi, A. and Chao, M. V. (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc. Natl. Acad. Sci. U S A.* 90: 7859-7863.

Berkemeier, L. R., Ozcelik, T., Francke, U., and Rosenthal, A. (1992). Human chromosome 19 contains the neurotrophin-5 gene locus and three related genes that may encode novel acidic neurotrophins. Somat Cell Mol Genet. 18:233-245

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V. and Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* 7: 857-866.

Bertollini, L., Ciotti, M. T., Cherubini, E., and Cattaneo, A. (1997). Neurotrophin-3 promotes the survival of oligodendrocyte precursors in embryonic hippocampal cultures under chemically defined conditions. Brain Res. 746:19-24.

Birchmeier, C. and Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol.* 8: 404-410.

Birchmeier, W., Brinkmann, V., Niemann, C., Meiners, S., DiCesare, S., Naundorf, H., and Sachs, M. (1997). Role of HGF/SF and c-Met in morphogenesis and metastasis of epithelial cells. *Ciba Found. Symp.* 212: 230-240.

Bjerre, B., Bjorklund, A., Mobley, W., and Rosengren, E. (1975a). Short- and long-term effects of nerve growth factor on the sympathetic nervous system in the adult mouse. Brain Res. 2: 263-277

Bjerre, B., Wiklund, L., and Edwards, D. C.(1975). A study of the de- and regenerative changes in the sympathetic nervous system of the adult mouse after treatment with the antiserum to nerve growth factor. Brain Res. 2:257-278.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature *376*: 768-771.

Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C. and Comoglio, P. M. (1998). Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature. 391: 285-288.

Boccaccio, C., Gaudino, G., Gambarotta, G., Galimi, F., and Comoglio, P.M. (1994). Hepatocyte Growth Factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. J. Biol. Chem. 269:12846-12851.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 74:597-608.

Borset, M., Lien, E., Espevik, T., Helseth, E., Waage, A., and Sundan, A. (1996). Concomitant expression of hepatocyte growth factor and the receptor c-Met in human myeloma cell lines. J. Biol. Chem. 271: 24655-24661.

Bothwell, M. (1991). Tissue localization of nerve growth factor and nerve growth factor receptors. *Curr Top Microbiol Immunol* 165: 55-70.

Bottaro, D. P., Rubin, J. S., Faletto, D, L., Chan, A. M. I., Kmiecik, T, E., Vande-Woude, G. F., and Aaronson, S. A. (1991). Identification of Hepatocyte Growth Factor receptor as the c-met proto-oncogene product. Science 251: 802-804.

Boulton, T. G., Stahl, N., and Yancopoulos, G. D. (1994). Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines andgrowth factors. *J. Biol. Chem.* 269: 11648-11655.

Bovolenta, P., Frade, J. M., Marti, E., Rodriguez-Pena, M. A., Barde, Y. A., and Rodriguez-Tebar.(1996). Neurotrophin-3 antibodies disrupt the normal development of the chick retina. J Neurosci. 16:4402-4410.

Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., and Lutz, R. J. (1995). Bik, a novel death-inducing protein shares a distinctsequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene. 11:1921-1928.

Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. (1995). Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J. Cell Biol. *131*: 1573-1586.

Buchman, V. L. and Davies, A. M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development* 118: 989-1001.

Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A., and Davies, A. M. (1995). GDNF is an age-specific survival factor for sensory and automomic neurons. Neuron 15: 821-828.

Buj-Bello, A., Adu, J., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V. L., and Davies, A. M. (1997). Neurtrin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 387: 721-724.

Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., and Li, P. (1995). Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science, 269: 1885-1888.

Bussolino, F., Di-Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagone, L., Coffer, A., and Comoglio, P. M. (1992). Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. 119: 629-641.

Buttke, T. M., and Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis Immunol Today. 157-10.

Carr, P. A., and Nagy, J. I. (1993). Emerging relationships between cytochemical properties and sensory modality transmission in primary sensory neurons. Brain Res Bull. 30: 209-219

Carroll, P., Sendtner, M., Meyer, M. and Thoenen, H. (1993). Rat ciliary neurotrophic factor (CNTF): gene structure and regulation of mRNA levels in glial cell cultures. *Glia* 9: 176-187.

Carroll, S. L., Silos-Santiago, I., Frese, S. E., Ruit, K..G., Milbrandt, J., and Snider, W. D. (1992). Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero. Neuron. 9:779-788

Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T. and Chao, M. V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* 383: 716-719.

Cavicchioli, L., Flanigan, T. P., Vantini, G., Fusco, M., Polato, P., Toffano, G., Walsh, F. S. and Leon, A. (1989). NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. *Eur. J. Neurosci.* 1: 258-262.

Chao, M. V. (1992). Growth factor signaling: where is the specificity? *Cell* 68: 995-997.

Chao, M. V. (1994). The p75 neurotrophin receptor. *J. Neurobiol.* 25: 1373-1385.

Chao, M. V., and Hempstead, B. L. (1995) p75 and Trk: a two-receptor system. Trends Neurosci. 18:321-326.

Chao, M. V., Bothwell, M. A., Ross, A. H., Koprowski, H., Lanahan, A. A., Buck, C. R. and Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. *Science* 232: 518-521.

Chen, C. Y., and Faller, D. V.(1996). Phosphorylation of Bcl-2 protein and association with p21Ras in Ras-induced apoptosis. J Biol Chem. 271:2376-2379.

Chen, D. F., Schneider, G. E., Martinou, J. C., and Tonegawa , S. (1997). Bcl-2 promotes regeneration of severed axons in mammalian CNS. *Nature* 385:434-439.

Chen-Levy, Z., Nourse, J., and Cleary, M. L. (1989). The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol. Cell.Biol.. 9:701-710.

Chesa, P. G., Rettig, W. J., Thomson, T. M., Old, L. J. and Melamed, M. R. (1988). Immunohistochemical analysis of nerve growth factor receptor expression in normal and malignant human tissues. *J Histochem Cytochem* 36: 383-389.

Chinnaiyan, A. M., and Dixit, V. M. (1996). The cell-death machine.Current Biology. 6: 555-562.

Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz R, J., Evan, G., and Guild, B. C. (1995). Induction of apoptosis by the Bcl-2 homologue Bak. Nature. 374:733-736.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*: 156-159.

Chou-Lee, C., and Yamada, K. M. (1995). Alternatively spliced juxtamembrane domain of a tyrosine kinase receptor is a multifunctional regulatory site. J. Biol. Chem. 270: 507-510.

Clary, D. O. and Reichardt, L. F. (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin-3. *Proc. Natl. Acad. Sci. USA.* 91: 11133-11137.

Clatterbuck, R. E., Price, D. L., and Koliatsos, V. E. (1993). Ciliary neurotrophic factor prevents retrograde neuronal death in the adult central nervous system. Proc. Natl. Acad. Sci. U S A. 90:2222-2226.

Cleary, M. L., and Skla, R. J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. U S A.82:7439-7443.

Cohen, A. M. (1974). DNA synthesis and cell division in differentiating avian adrenergic neuroblasts. In K. Fuxe, L. Olson, & Y. Zotterman (Eds.), *Wenner-Gren Center International Symposium Series: Dynamics of Degeneration and Growth in Neurons.* Oxford: Pergamon.

Cohen, A., Bray, G. M. and Aguayo, A. J. (1994). Neurotrophin-4/5 (NT-4/5) Increases Adult Rat Retinal Ganglion Cell Survival and Neurite Outgrowth in vitro. *J. Neurobiol.* 25: 953-959.

Cohen, J. J., and Duke, R. C. (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol. 132:38-42.

Cohen, S. (1959). *J. Biol. Chem.* 234: 1129-1137.

Cohen, S. (1960). Purification of nerve growth factor-promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. *Proc.. Natl.. Acad.. Sci..* 46: 302-311.

Cohen-Cory, S., and Fraser, S. E. (1995) .Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature. 378:192-6

Collazo, D., Takahashi, H. and McKay, R. D. (1992). Cellular targets and trophic functions of neurotrophin-3 in the developing rat hippocampus. *Neuron* 9: 643-56.

Conner, E. A. Wirth, P. J., Kiss, A., Santoni-Rugiu, E., and Thorgeirsson, SS. (1997). Growth inhibition and induction of apoptosis by HGF in transformed rat liver epithelial cells. Biochem. Biophys. Res. Comm. 236: 396-401.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeClara, T. M. and Yancopoulas, G. D. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and NT4. *Nature* 375: 235-238.

Cooper, C. S., Park, M., Blair, M. A., Tainsky, K., Huebner, C. M., Croce, C., and Vande woude, G. F. (1984). Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature 311: 29-33.

Cooper, C. S., Tempest, P. R., Beckman, M.P., Heldin, C.H., and Brookes, P. (1986). Amplification and overexpression of the met gene in spontaneously transformed NIH3T3 mouse fibroblasts. EMBO. J. 10: 2623-2623-8.

Cordon-Cardo, C., Tapley, P., Jing, S. Q., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K. R., Reichardt, L. F. and Barbacid, M. (1991). The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell* 66: 173-183.

Crepaldi, T., Pollack, A. L. Prat, M., Zborek, A., Mostov, K., and Comoglio, P. M. (1994a). Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. J. Cell Biol. 125: 313-320.

Crepaldi, T., Prat, M., Giordano, S., Medico, E., and Comoglio, P. M. (1994b). Generation of a truncated Hepatocyte growth factor receptor in the Endoplasmic reticulum. J. Biol. Chem. 269: 1750-1755.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., S., P.-M., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D. and Phillips, H. S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* 76: 1001-1011.

Davey, F., and Davies, A. M. (1998). TrkB signalling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. Curr Biol. 8:915-918

Davies, A. M., and Lumsden, A. G. S (1984). Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol.* 223: 124-137.

Davies, A. M., Thoenen, H., and Barde, Y. A. (1986a). Different factors from the central nervous system and periphery regulate the survival of sensory neurones.Nature. 319:497-499.

Davies, A. M, Thoenen, H., and Barde, Y. A. (1986b). The response of chick sensory neurons to brain-derived neurotrophic factor.J Neurosci. 6:1897-1904.

Davies, A. M. (1986). The survival and growth of embryonic proprioceptive neurons is promoted by a factor present in skeletal muscle. Dev Biol. 115:56-67.

Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987a). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature* 326: 353-358.

Davies, A. M. (1987b). Molecular and cellular aspects of patterning sensory neurone connections in the vertebrate nervous system. *Development* 101: 185-208.

Davies, A. M., Lumsden, A. G. and Rohrer, H. (1987c). Neural crest-derived proprioceptive neurons express nerve growth factor receptors but are not supported by nerve growth factor in culture. *Neuroscience* 20: 37-46.

Davies, A. M. (1988a). The emerging generality of the neurotrophic hypothesis. Trends Neurosci. 11: 243-247.

Davies, A. M. (1988b). Role of neurotrophic factors in development. Trends Genet. 4:139-143.

Davies, A. M. (1989). Intrinsic differences in the growth rate of early nerve fibres related to target distance. Nature *337*: 553-555.

Davies, A. M., Horton, A., Burton, L. E., Schmelzer, C., Vandlen, R. and Rosenthal, A. (1993a). Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. *J. Neurosci.* 13: 4961-4967.

Davies, A. M., Lee, K. F. and Jaenisch, R. (1993b). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* 11: 565-74.

Davies, A. M. (1994). Chemoattractants for navigating axons. Curr. Biol. 4: 1142-1145.

Davies, A. M. (1994a). Role of neurotrophins in the developing nervous system. *J. Neurobiol.* 25: 1334-1348.

Davies, A. M. (1994b). Tracking neurotrophin function. *Nature* 368: 193-194.

Davies, A. M. (1995). The Bcl-2 family of proteins, and the regulation of neuronal survival. Trends Neurosci. 18:355-358.

Davies, A. M., Minichiello, L., and Klein, R. (1995). Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. EMBO J. *14*: :4482-4489.

Davies, A. M., Wyatt, S., Nishimura, M. and Phillips, H. (1995c). NGF receptor expression in sensory neurons develops normally in embryos lacking NGF. *Devel. Biol.* 171: 434-438.

Davies, A. M. (1997). Neurotrophin switching: where does it stand. Curr. Opin. Neurobiol. *7*: 110-118.

Davis, S., Aldrich, T. H., Ip, N. Y., Stahl, N., Scherer, S., Farruggella, T., DiStefano, P. S., Curtis, R., Panayotatos, N., Gascan, H., Chelalier, S., and Yancopoulos, G. D. (1993). Released form of CNTF receptor alpha component as a soluble mediator of CNTF responses. *Science* 259: 1736-1739.

Dean, M., Park, M., Le-Beau, M. M., Robins, T. S., Daiz. M. O., Rowley, J, D., Blair, D. G., and Vande-woude, F. G. (1985). The human met oncogene is related to the tyrosine kinase oncogenes. Nature 318: 385-388.

Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, E. M. Jr., Snider, W. D., and Korsmeyer, S. J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron. 17:401-411

DeFrances, M. C., Wolf, H. K., Michalopoulos, G. K.., and Zarnegar, R. (1992). The presence of hepatocyte growth factor in the developing rat. Development 116: 387-395.

De-Jaun, C., Sanchez, A., Nakamura, T., Fabregat, I., and Benito, M. (1994). Hepatocyte Growth Factor up-regulates met expression in rat fetal hepatocytes in primary culture. Biochem. Biophys. Res. Comm. 204: 1364-1369.

Depuydt, C. E. Zalata, A., De-Potter, C. R., Van-Emmelo, J., Comhaire, F.H. (1996). The receptor encoded by the human C-MET oncogene is expressed in testicular tissue and on human spermatozoa. Mol. Hum. Reprod. 2: 2-8.

DiCicco-Bloom, E., and Black, I. B. (1988). Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. Proc. Natl. Acad. Sci. USA *85*: 4066-4070.

DiCicco-Bloom, E., Townes-Anderson, E., and Black, I. B. (1990). Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. J. Cell Biol. *110*: 2073-2086.

DiCicco-Bloom, E., Friedman, W. J. and Black, I. B. (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor cell survival. *Neuron* 11: 1101-1111.

Dijkhuizen, P. A., Hermens, W. T., Teunis, M. A., and Verhaagen, J. (1997). Adenoviral vector-directed expression of neurotrophin-3 in rat dorsal root ganglion explants results in a robust neurite outgrowth response. J Neurobiol. 33:172-184

Di-Renzo, M. F., Narsimhan, R. P., Olivero, M., Bretti, S., Giordano,S., Medico, E., Gaglia, P., Zara, P., and Comoglio, P.M. (1991). Expression of the Met/HGF receptor in normal and neoplastic human tissues. Oncogene 6: 1997-2003.

Di-Renzo, M. F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Costantino, A., Vigneri, R., and Pierotti, M. A. (1992). Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. Oncogene 12: 2549-2553.

Di-Renzo, M. F., Bertolotto, A., Olivero, M., Putzolu, P., Crepaldi, T., Schiffer, D., Pagni, C. A., and Comoglio, P. M. (1993). Selective expression of the Met/HGF receptor in human central nervous system microglia. *Oncogene* 8: 219-222.

DiStefano, P. S. and Johnson, E. M. J. (1988). Identification of a truncated form of the nerve growth factor receptor. *Proc. Natl. Acad. Sci. U S A.* 85: 270-274.

Dobrea, G. M., Unnerstall, J. R. and Rao, M. S. (1992). The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. *Devel. Brain Res.* 66: 209-219.

Duan, H., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W, and Dixit, V. M. (1996). ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell-death protease Ced-3 is activated during FAS- and tumour necrosis factor-induced apoptosis. J. Biol. Chem. 271: 1621-1625.

Dubois-Dauphin, M., Frankowski, H., Tsujimoto, Y., Huarte, J., and Martinou, J. C. (1994). Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. U S A. 91:3309-3313.

Durkop, H., Latza, U., Hummel, M., Eitelbach, F., Seed, B., and Stein, H. (1992). Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. Cell. 68:421-427.

Ebendal, T., Jordell-Kylberg, A., and Soderstorm, S. (1978). Stimulation by tissue explants on nerve fiber outgrowth in culture. Zoon 6: 235-243.

Ebendal, T., Larhammar, D. and Persson, H. (1986). Structure and expression of the chicken beta nerve growth factor gene. *Embo J.* 5: 1483-7.

Ebens, A., Brose, K., Leonardo, E. D., Hanson, M. G. J., Bladt, F., Birchmeier, C., Barres, B. A., and Tessier-Lavigne, M. (1996). Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron *17*, 1157-1172.

Edwards, R. H., Selby, M. J., and Rutter, W. J. (1986). Differential RNA splicing predicts two distinct nerve growth factor precursors. Nature.319: 784-787.

Ehrhard, P. B., Erb, P., Graumann, U. and Otten, U. (1993). Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc. Natl. Acad. Sci. U S A.* 90: 10984-10988.

Ellis, H. M., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell. 44:817-829.

ElShamy, W. M. and Ernfors, P. (1996). A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. *Neuron* 16: 963-972.

Elshamy, W. M., and Ernfors, P. (1996). Requirement of neurotrophin-3 for the survival of proliferating trigeminal ganglion progenitor cells. Development. 122:2405-2414.

ElShamy, W. M., and Ernfors, P. (1996). A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. Neuron *16*: 963-972. EMBO J. *16*: 3115-3123.

Enokido, Y., de Sauvage, F., Hongo, J.-A., Ninkina, N., Rosenthal, A., Buchman, V. L., and Davies, A. M. (1998). GFRA4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr. Biol. 8: 1019-1022.

Epstein, J.A., Shapiro, D,N., Cheng, J., Lam, P. Y P., and Maas, R. L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. *PNAS* 93: 4213-4218.

Erickson, J. T., Conover, J. C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G. and Katz, D. M. (1996). Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J. Neurosci.* 16: 5361-5371.

Eriksson, N. P., Lindsay, R. M. and Aldskogius, H. (1994). BDNF and NT-3 rescue sonsory but not motoneurones following axotomy in the neonate. *NeuroReport* 5: 1445-1448.

Ernfors, P., Hallbook, F., Ebendal, T., Shooter, E. M., Radeke, M. J., Misko, T. P. and Persson, H. (1988). Developmental and regional expression of beta-nerve growth factor receptor mRNA in the chick and rat. . *Neuron* 1: 983-996.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L. and Persson, H. (1990a). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA.* 87: 5454-5458.

Ernfors, P., Wetmore, C., Olson, L. and Persson, H. (1990b). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 5: 511-526.

Ernfors, P., Merlio, J. and Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Euro. J. Neurosci.* 4: 1140-1158.

Ernfors, P., Lee, K. F. and Jaenisch, R. (1994a). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 368: 147-150.

Ernfors, P., Lee, K. F., Kucera, J. and Jaenisch, R. (1994b). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* 77: 503-512.

Ernfors, P., Kucera, J., Lee, K. F., Loring, J., and Jaenisch, R. (1995). Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. Int J Dev Biol. 39:799-807.

Ernsberger, U., Edgar, D., and Rohrer, H. (1989a). The survival of early chick sympathetic neurons in vitro is dependent on a suitable substrate but independent of NGF. Devel. Biol. *135*: 250-262.

Ernsberger, U., Sendtner, M., and Rohrer, H. (1989b). Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. Neuron 2: 1275-84.

Evans, B. A., and Richards, R. I. (1985). Genes for the alpha and gamma subunits of mouse nerve growth factor are contiguous. EMBO. J. 4: 133-138.

Fagan, A. M., Zhang, H., Landis, S., Smeyne, R. J., Silos-Santiago, I., and Barbacid, M. (1996). TrkA, but not trkC, receptors are essential for survival of sympathetic neurons in vivo. J. Neurosci. *16*: 6208-6218.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y. and Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* 369: 658-661.

Farinas, I., Wilkinson, G. A., Backus, C., Reichardt, L. F., and Patapoutian, A. (1998). Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo. Neuron. 21:325-334.

Farinas, I., Yoshida, C. K., Backus, C., and Reichardt, L. F.(1996). Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron. 17:1065-78

Farlie, P. G., Dringen, R., Rees, S. M., Kannourakis, G., and Bernard, O. (1995). bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc. Natl. Acad. Sci. U S A. 92:4397-4401.

Faucheu, C., Diu, A., Chan, A. W., Blanchet, A. M., Miossec, C., and Herve, F.(1995). A novel human protease similar to interleukin-1 beta converting enzyme induces apoptosis in transfected cells. EMBO. J. 14: 1914-1922.

Fernandez-Sarabia, M. J., and Bischoff, J. R. (1993). Bcl-2 associates with the ras-related protein R-ras p23. Nature. 366:274-275.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994). CPP32, a novel human apoptotic protein with homology to Caenorhadbitis elegans cell death protein Ced-3 and mammalian interleukin-1b-converting enzyme. J. Biol. Chem. 269: 30761-30764.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995a). Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res, 55: 2737-2742.

Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., and Tomaselli, K. J. (1995b). Mch3, a novel human apoptotic cysteine protease highly related to CPP32. Cancer Res. 55: 6045-6052.

Ferracini, R., Longati, P., Naldini, L., Vigna, E., and Comoglio, P. M. (1991). Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J. Biol. Chem. 266: 19558-19564.

Finn, T. P., Kim, S., and Nishi, R. (1998). Overexpression of ciliary neurotrophic factor in vivo rescues chick ciliary ganglion neurons from cell death. J Neurobiol. 34:283-293.

Fitzgerald, M. Kwait, G. C., Middleton, J., and Pini, A. (1993). Ventral spinal cord inhibition of neurite outgrowth from embryonic rat dorsal root ganglia. Development 117: 1377-1384.

Fixman, E. D., Fournier, T, M., Kamikura, D. M., Naujokas, M. A., and Park, M. (1996). Pathways down stream of Shc and Grb2 are required for cell transformation by the tpr-met oncoprotein. J. Biol. Chem. 271: 13116-13122.

Flanagan, J. G., and Vanderhaegen, P. (1998). The eph receptors in neural development. Ann. Rev. Neurosci. 21: 309-345.

Forscher, P., Lin, C. H., and Thompson, C. (1992). Novel form of growth cone motility involving site-directed actin filament assembly. Nature 357: 515-518.

Frade, J. M., and Barde, Y. A. (1999). Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development. 126:683-690.

Frade, J. M., Rodriguez-Tebar, A. and Barde, Y. A. (1996). Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* 383: 166-168.

Francis, N., Farinas, I., Brennan, C., Rivas-Plata, K., Backus, C., Reichardt, L., and Landis, S. (1999). NT-3, like NGF, is required for survival of sympathetic neurons, but not theirprecursors. Dev Biol. 210:411-427.

Frank, D. A. and Greenberg, M. E. (1996). Signal transduction pathways activated by ciliary neurotrophic factor and related cytokines. *Perspect. Dev. Neurobiol.* 4: 3-18.

Franz, T., Kothary, R.., Surani, M. A., Halata, Z., and Grim, M. (1993). The Splotch mutation interferes with muscle development in the limbs. *Anat-Embryol-Berl.* **187**: 153-160.

Friedman, B., Kleinfeld, D., Ip, N. Y., Verge, V. M., Moulton, R., Boland, P., Zlotchenko, E., Lindsay, R. M., and Liu, L. (1995). BDNF and NT-4/5 exert neurotrophic influences on injured adult spinal motor neurons. J Neurosci. 15:1044-1056.

Fritzsch, B., Farinas, I., and Reichardt, L. F. (1997). Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. J Neurosci. 17:6213-25

Fukuyama, R., Ichijoh, Y., Minoshima, S., Kitamura, N., and Shimizu, N. (1991). Regional localization of the hepatocyte growth factor (HGF) gene to human chromosome 7 band q21.1. Genomics 11: 410-415.

Funakoshi, H., Belluardo, N., Arenas, E., Yamamoto, Y., Casabona, A., Persson, H. and Ibanez, C. F. (1995). Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. *Science* 268: 1495-1499.

Furlong, R. Takehara, T. Taylor, W. T., Nakamura, T., and Rubin, J.S.(1991). Comparison of biological and immunochemical properties indicates that scatter factor and hepatocyte growth factor are indistinguishable. J. Cell. Sci 100: 173-177.

Gaese, F., Kolbeck, R. and Barde, Y. A. (1994). Sensory ganglia require neurotrophin-3 early in development. *Development* 120: 1613-1619.

Gambarotta, G., Pistoi, S., Giordano, S., Comoglio, P. M., and Santoro, C. (1994). Structure and inducible regulation of the human MET promoter. J. Biol. Chem. 269: 12852-12857.

Gandino, L., Di-Renzo, M. F., Giordano, S., Bussolino, F., and Comoglio, P. M. (1990). Protein kinase-c activation inhibits tyrosine phosphorylation of the c-met protein. Oncogene 5: 721-725.

Gandino, L., Munaron, L., Naldini, L., Ferracini, R., Magni, M. and Comoglio, P.M. (1991). Intracellular calcium regulates the tyrosine kinase receptor encoded by the Met oncogene. J. Biol. Chem. 266: 16098-16104.

Gandino, L., Longati, P., Medico, E., Prat, M., and Comoglio, P. M. (1994). Phosphorylation or serine 985 negatively regulates the hepatocyte growth factor receptor kinase. J. Biol .Chem. 269: 1815-1820.

Gao, W. Q., Zheng, J. L. and Karihaloo, M. (1995). Neurotrophin-4/5 (NT-4/5) and brain-derived neurotrophic factor (BDNF) act at later stages of cerebellar granule cell differentiation. *J. Neurosci.* 15: 2656-2667.

Garcia, I., Martinou, I., Tsujimoto, Y., and Martinou, J. C. (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science* 258: 302-304.

Garner, A. S. and Large, T. H. (1994). Isoforms of the avian TrkC receptor: a novel kinase insertion dissociates transformation and process outgrowth from survival. *Neuron* 13: 457-472.

Gherardi, E., Gray,J., Stoker, M., Perryman, M., and Furlong, R. (1989). Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. Proc. Natl. Acad. Sci. USA, 86:5844-5848.

Gherardi, E., and Stoker, M. (1990). Hepatocytes and scatter factor. Nature 346: 228-231.

Gherardi, E., and Stoker, M. (1991). Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. Cancer Cells 3: 227-232.

Gibson, L., Holmgreen, S. P., Huang, D. C., Bernard, O., Copeland, N. G., Jenkins, N. A., Sutherland, G.R., Baker, E., Adams, J. M., and Cory, S. (1996). bcl-w, a novel member of the bcl-2 family, promotes cellsurvival. Oncogene. 13: 665-675.

Gillardon, F., Zimmermann, M., Uhlmann, E., Krajewski, S., Reed, J. C., and Klimaschewski, L. (1996). Antisense oligodeoxynucleotides to bax mRNA promote survival of rat sympathetic neurons in culture. J Neurosci Res. 43:726-734

Giordano, S., Di-Renzo, M. F., Narsimhan, R.. P., Tamagnone, L., Gerbaudo, E. V., Chiado-Piat, L., and Comoglio, P. M. (1988). Evidence for autocrine activation of a tyrosine kinase in a human gastric carcinoma cell line. J. Cell. Biochem. 38: 229-236.

Giordano, S., Ponzetto, C., Di-Renzo, M. F., Cooper, C. S., and Comoglio, P. M. (1989b). Tyrosine kinase receptor indistinguishable from the c-met protein. Nature 339: 155-156.

Giordano, S., Di-Renzo, M. F., Narsimhan, R. P., Cooper, C. S., Rosa, C., and Comoglio, P. M. (1989a). Biosynthesis of the protein encoded by the c-met proto-oncogene. Oncogene 4: 1383-1388.

Giordano, S., Di-Renzo, M. F., Olivero, M., Mondino, A., Zhen, Z., Medico, E., Comoglio, P. M. (1992). The c-met/HGF receptor in human tumours. Eur. J. Cancer. Prev. Suppl 3: 45-49.

Giordano, S., Zhen, Z., Medico, E., Gaudino, G., Galimi, F., and Comoglio, P. M. (1993). Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human Met protooncogene. Proc. Natl. Acad. Sci. USA, 90: 649-653.

Gohda, E., Tsubouchi, H., Nakayama, S., Hirono, O., Sakiyama, K., Takahashi, H., Miyazaki, S., Hasimoto, S., and Daikuhara, Y. (1988). Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J. Clin. Invest. 81: 414-419.

Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S., and Vande woude, G.F. (1988). Characterization of the TPR-MET oncogene p65 and the MET protooncogene p140 protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA. 85: 21-25.

Goodman, C.S., and C.J. Shatz. 1993. Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell/Neuron (Suppl.)* 72/10: 77-98.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M. and Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* 372: 266-269.

Graziani, A., Gramaglia, D., Cantley, L. C., and Comoglio, P. M. (1991). The tyrosine-phosphorylated Hepatocyte Growth Factor/Scatter Factor receptor associates with phosphatidylinositol 3-kinase. J. Biol. Chem. 266: 22087-22090.

Graziani, A., Gramaglia, D., Zonca, P, D., and Comoglio, P. M. (1992). Hepatocyte Growth Factor / Scatter Factor stimulates the Ras-Guanine nucleotide exchanger. J. Biol. Chem. 268: 9165-9168.

Greene, L. A., Shooter, E. M., and Varon, S. (1969). Subunit interaction and enzymatic activity of mouse 7S nerve growth factor. Biochemistry. 8: 3735-3741.

Greene, L. A. and Shooter, E. M. (1980). The nerve growth factor: biochemistry, synthesis, and mechanism of action. *Annu Rev Neurosci* 3: 353-402.

Greene, L. A., and Kaplan, D. R. (1995). Early events in neurotrophin signalling via Trk and p75 receptors.Curr. Opin. Neurobiol.579-587.

Greenlund, L. J., Korsmeyer, S.J , and Johnson, E. M. (1995). Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. *Neuron* 15:649-661.

Hagg, T., and Varon, S. (1993). Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. Proc. Natl. Acad. Sci. U S A. 90: 6315-6319

Hall, A. (1990). The cellular functions of small GTP-binding proteins. Science 249: 635-640.

Hallbook, F., Ayer, L. C., Ebendal, T. and Persson, H. (1990). Expression of nerve growth factor receptor mRNA during early development of the chicken embryo: emphasis on cranial ganglia. *Development* 108: 693-704.

Hallbook, F., Ibanez, C. F. and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron* 6: 845-858.

Hallbook, F., Ibanez, C. F., Ebendal, T. and Persson, H. (1993). Cellular localization of brain-derived neurotrophic factor and neurotrophin-3 mRNA expression in the early chicken embryo. *Eur. J. Neurosci.* 5: 1-14.

Hama, T., Miyamoto, M., Tsukui, H., Nishio, C. and Hatanaka, H. (1989). Interleukin-6 as a neurotrophic factor for promoting the survival of cultured basal forebrain cholinergic neurons from postnatal rats. *Neurosci. Lett.* 104: 340-4.

Hamanoue, M., Takemoto, N., Matsumoto, K., Nakamura, T., Nakajima, K., and Kohsaka, S.(1996), Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J. Neurosci. Res. 43: 554-564.

Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D., and White, E. (1996). The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev. 10:461-477.

Harper, G. P., Barde, Y. A., Burnstock, G., Carstairs, J. R., Dennison, M. E., Suda, K., and Vernon, C. A. (1979). Guinea pig prostate is a rich source of nerve growth factor. Nature. 279: 160-162.

Harper, G. P., and Thoenen, H. J. (1980). The distribution of nerve growth factor in the male sex organs of mammals. Neurochem. 4:893-903

Harper, S. and Davies, A. M. (1990). NGF mRNA expression in developing cutaneous epithelium related to innervation density. *Development* 110: 515-519.

Harris, W. A., and Holt, C. E. (1995). From tags to RAGS: chemoaffinity finally has receptor and ligands. Neuron 15, 241-244.

Hartikka, J., and Hefti, F. (1988a). Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons in vitro.J Neurosci Res 1988 21:352-364.

Hartikka, J. and Hefti, F. (1988b). Development of septal cholinergic neurons in culture: plating density and glial cells modulate effects of NGF on survival, fiber growth, and expression of transmitter-specific enzymes. J Neurosci. 8:2967-2985.

Hartmann, G., Naldini, L., Weidner, K. M., Sachs, M., Vigna, E., Comoglio, P. M., and Birchmeier, W. (1992). A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-met receptor and induces cell dissociation but not mitogenesis. Proc. Natl. Acad. Sci. USA 89: 11574-11578.

Hearn, C. J., Murphy, M., and Newgreen, D. (1998). GDNF and ET-3 differentially modulates the number of avian enteric neural crest cells and enteric neurons in vitro. Dev. Biol. 197: 93-105.

Helfand, S. L., Smith, G. A., and Wessells, N. K. (1976). Survival and development of dissociated parasympathetic neurons from ciliary ganglia. *Dev. Biol.* 50: 541-547.

Hempstead, B. L., Martin, Z. D., Kaplan, D. R., Parada, L. F. and Chao, M. V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* 350: 678-683.

Henderson, C. E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karlaloo, M., Rullamas, J., Evans, T., McMahon, S. B., Armanini, M. P., Berkemeier, L., Phillips, H. S. and Rosenthal, A. (1993). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature* 363: 266-270.

Henderson, C.E. 1996. Role of neurotrophic factors in neuronal development. *Current Opinion in Neurobiology* **6**: 64-70.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein-Barr virus-coded BHRF1 protein, a viralhomologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci. U S A. 90: 8479-8483.

Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature. 356:494-499.

Hengartner, M. O., and Horvitz, H. R. (1994) Programmed cell death in Caenorhabditis elegans. Curr Opin Genet Dev. 4: 581-586.

Heuckeroth, R. O., Lampe, P. A., Johnson, E. M. J., and Milbrandt, J. (1998). Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev. Biol. 200: 116-129.

Heuckeroth, R. O., Enomoto, H., Grider, J. R., Golden, J. P., Hanke, J. A., Jackman, A., Molliver, D. C., Bardgett, M. E., Snider, W. D., Johnson, E. M. J., and Milbrandt, J. (1999). Gene Targeting Reveals a Critical Role for Neurturin in the Development and Maintenance of Enteric, Sensory, and Parasympathetic Neurons. Neuron, 22: 253-263.

Heuer, J. G., Fatemie-Nainie, S., Wheeler, E. and Bothwell, M. (1990). Structure and developmental expression of the chicken NGF receptor. *137:* 287-304.

Heumann, R., Korsching, S., Scott, J. and Thoenen, H. (1984). Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *Embo J.* 3: 3183-3189.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kashimoto, T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell, 63: 1149-1157.

Higashio, K., Shima, K., Goto, M., Itagaki, Y., Nagao, M., Yasuda, H., and Morinaga, T. (1990). Identity of a tumor cytotoxic factor from human Fibroblasts and Hepatocyte Growth Factor. Biochem. Biophys. Res. Comm. 170: 397-404.

Higuchi, O., Mizuno, K., Vande woude, G. F., and Nakamura, T. (1992). Expression of c-met proto-oncogene in COS cell induces the signal transducing high affinity receptor for hepatocyte growth factor. FEBS Lett 301: 282-286.

Hilton, M., Middleton, G., and Davies, A. M. (1997). Bcl-2 influences axonal growth rate in embryonic sensory neurons. Curr. Biol. 7: 798-800.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 348:334-346.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L, and Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell. 75:241-251.

Hofer, M. M. and Barde, Y. A. (1988). Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* 331: 261-262.

Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J., and Barde, Y.A. (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J. 9:2459-2464.

Hohn, A., Leibrock, J., Bailey, K. and Barde, Y. A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* 344: 339-341.

Honda, S., Kagoshima, M., Wanaka, A., Tohyama, M., Matsumoto,K., Nakamura, K. (1995). Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: Implication as neurotrophic factor. Mol. Brain. Res. 32: 197-210.

Horton, A. R., Bartlett, P. F., Pennica, D. and Davies, A. M. (1998). Neurotrophic cytokines promote the survival of cranial sensory neurons at different stages of their development. Eur. J. Neurosci. 10: 673-679.

Hory-Lee, F., Russell, M., Lindsay, R. M. and Frank, E. (1993). Neurotrophin 3 supports the survival of developing muscle sensory neurons in culture. *Proc. Natl. Acad. Sci. U S A.* 90: 2613-26137.

Hsu, S.Y, Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A. J. (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc. Natl. Acad. Sci. U S A. 94:12401-12406.

Hu, Y., Benedict, M. A., Wu, D., Inohara, N., and Nunez, G. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci U S A. 95:4386-4391

Huang, E. J., Wilkinson, G. A., Farinas, I., Backus, C., Zang, K., Wong, S. L., and Reichardt, L. F. (1999). Expression of trk receptors in the developing mouse trigeminal ganglion: in vivo evidence for NT-3 activation of TrkA and TrkB in addition to trkC. Development.126: 2191-203.

Huff, J.L., Jelinek, M. A., Borgman, C. A., Lansing., and Parons, J. T. (1993). The protooncogene c-sea encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. Proc. Natl. Acad. Sci. USA 90: 6140-6144.

Hyman, C., Hofer, M., Barde, Y. A., Juhasz, M., Yancopoulos, G. D., Squinto, S. P. and Lindsay, R. M. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 350: 230-232.

Hynes, R. O., and Lander, A.D.(1992). Contact and adhesive specificity's in the associations, migrations, and targeting of cells and axons. Cell 68: 303-22.

Ibanez, C. F., Hallbook, F., Ebendal, T. and Persson, H. (1990). Structure-function studies of nerve growth factor: functional importance of highly conserved amino acid residues. *EMBO J.* 9: 1477-1483.

Ibanez, C. F., Ernfors, P., Timmusk, T., Ip, N. Y., Arenas, E., Yancopoulos, G. D. and Persson, H. (1993). Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development* 117: 1345-53.

Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y., and Nakamura, T.(1991). Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem. Biophys. Res. Comm. 174: 831-838.

Igawa, T., Matsumoto, K., Kanda, S., Saito, Y., and Nakamura, T. (1993). hepatocyte Growth Factor may function as a renotropic factor for regeneration in rats with acute renal injury. Am. J. Physiol. F61-F69.

Indo, Y., Tsuruta, M., Hayashida, Y., Karim, M. A., Ohta, K., Kawano, T., Mitsubuchi, H., Tonoki, H., Awaya, Y., and Matsuda, I. (1996). Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis.

Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997). harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L). EMBO J. 16:1686-1694.

Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Merry, A., Chen, S., and Nunez, G. (1998). Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL. J Biol Chem. 273: 8705-8710.

Ip, N. Y., Li, Y. P., van, d. S. I., Panayotatos, N., Alderson, R. F. and Lindsay, R. M. (1991). Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. *J Neurosci* 11: 3124-3134.

Ip, N. Y., Ibanez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., Le, B. M., Espinosa, R. 3., Squinto, S. P., Persson, H. and Yancopoulas, G. D. (1992a). Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. U S A* 89: 3060-3064.

Ip, N. Y., Li, Y., Yancopoulos, G. D. and Lindsay, R. M. (1993a). Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. *J Neurosci* 13: 3394-405.

Ishibashi, K., Sasaki, S., Sakamoto, H., Nakamura, Y., Hata, T., Nakamura, T., and Marumo, F. (1992). Hepatocyte Growth Factor is a paracrine factor for renal epithelial cells: stimulated of DNA synthesis and NA, K-ATPase activity. Biochem. Biophys. Res. Comm. 182: 960-965.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell. 66:233-243.

Iyer, A., Kmiecik, T.E., Park, M., Daar, I., Blair, D., Dunn, K. J., Sutrave, P., Ihle, J.N., Bodescot, M., and Vande woude, G. F. (1990). Structure, tissue specific expression, and transforming activity of mouse met prootoncogene. Cell. Growth. Differ. 2: 87-95.

Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature. 361:365-369.

Jennische, E., Ekberg, S., and Matejka, G. L. (1993). Expression of hepatocyte growth factor in growing and regenerating rat skeletal muscle. Am. J. Physiol. C122-C128.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Lou, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., et al. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-a, a novel receptor for GDNF. Cell, 85: 1113-1124.

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P. L., Boone, T., Delaney, J., Schultz, H., Zhou, R., and Fox, G. M. (1997). GFRa-2 and GFRa-3 are two new receptors for ligands of the GDNF family. J. Biol. Chem. 272: 33111-33117.

Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. and Chao, M. (1986b). Expression and structure of the human NGF receptor. *Cell* 47: 545-54.

Johnson, E. M., Gorin, P. D., Brandeis, L. D., Pearson, J. 1980). Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. Science. 210: 916-918

Johnson, J. E., Barde, Y. A., Schwab, M. and Thoenen, H. (1986a). Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. *J Neurosci* 6: 3031-8.

Johnston, R. N., and Wessells, N. k. (1980). Regulation of the elongating nerve fiber: Current topics in developmental biology. Moscona, A.A., and Monray, A., (eds), 16: pp. 165-206. Newyork: Academic.

Jones, K. R. and Reichardt, L. F. (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. U S A* 87: 8060-8064.

Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* 76: 989-999.

Jung, W., Castren, E., Odenthal, M., Vande Woude, G. F., Ishii, T., Dienes, H. P., Lindholm, D., and Schirmacher, P. (1994). Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. J. Cell Biol. *126*: 485-494.

Junger, H., and Varon, S. (1997). Neurotrophin-4 (NT-4) and glial cell line-derived neurotrophic factor (GDNF) promote the survival of corticospinal motor neurons of neonatal rats in vitro. Brain Res. 762:56-60.

Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. (1995). bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. 55:354-359.

Kamens, J., Paskind, M., Hugunin, M., Talanian, R. V., Allen, H., and Banach, D. (1995). Identification and characterization of ICH-2, a novel member of the interleukin-1 beta-converting enzyme family of cysteine proteases. J. Biol. Chem. 270: 15250-15256.

Kan, M., Zhang, G, Zarnegar, R., Michalopoulos, G., Myoken, Y., McKeehan, W.L., and Stevens, J. (1991). Hepatocyte Growth Factor/Hepatopoitin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. Biochem. Biophys. Res. Comm. 174: 331-337.

Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord T., and Bredesen, D. E. (1993).Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science. 262:1274-1277.

Kaplan, D. R.., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991a). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science. 252: 554-558.

Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991b). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature. 350:158-160.

Kaplan, D. R., and Stephens, R. M.(1994). Neurotrophin signal transduction by the Trk receptor. J. Neurobiol. 25:1404-1417.

Kaplan, D. R., and Miller, F. D. (1997). Signal transduction by the neurotrophin receptors. Curr. Opin. Cell. Biol. 9:213-221

Kasai, S., Sugimura, K., Matsumoto, K., Nishi, N., Kishimoto, T., and Nakamura, T. (1996). Hepatocyte Growth Factor is a paracrine regulator of rat prostate epithelial growth. Biochem. Biophys. Res. Comm. 228: 646-652.

Kawaida, K., Matsumoto, K., Shimazu, H., and Nakamura, T., (1994). Hepatocyte Growth Factor prevents acute renal failure and accelerates renal regeneration. Proc. Natl. Acad. Sci. USA. 91: 4357-4361.

Kelekar, A., and Thompson, C. B.(1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. 8:324-330.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon withwide-ranging implications in tissue kinetics. *Br. J. Cancer* 26: 239-257.

Keynes, R. J., and Cook, G. M. W. (1995a). Repulsive and inhibitory signals. Curr.Opin. Neurobiol. 5: 75-82.

Kishimoto, T., Akira, S., and Taga, T. (1992). Interleukin-6 and its receptor: a paradigrm for cytokines. Science, 258: 593-597.

Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z. M., Saxena, S., Weichselbaum, R., Nalin, C., and Kufe, D. (1997). Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis.Proc Natl Acad Sci U S A. 94:6939-69342.

Klein, R., Parada, L. F., Coulier, F. and Barbacid, M. (1989). trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.* 8: 3701-3709.

Klein, R., Conway, D., Parada, L. F. and Barbacid, M. (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* 61: 647-656.

Klein, R., Jing, S. Q., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991a). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65: 189-197.

Klein, R., Lamballe, F., Bryant, S. and Barbacid, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* 8: 947-956.

Klein, R., Smeyne, R. J., Wurst, W., Long, L. K., Auerbach, B. A., Joyner, A. L. and Barbacid, M. (1993). Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* 75: 113-122.

Klein, R. (1994a). Role of neurotrophins in mouse neuronal development. *The FASEB Journal* 8: 738-744.

Klein, R., Silos, S. I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., Zhang, L., Snider, W. D. and Barbacid, M. (1994b). Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. *Nature* 368: 249-251.

Klein, R. D., Sherman, D., Ho, W. H., Stone, D., Bennett, G. I., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J. A.(1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature, 387: 717-721.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D.(1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 275:1132-1136.

Knusel, B., Michel, P.P., Schwaber, J.S., and Hefti, F. (1990). Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro bynerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. J Neurosci. 2:558-570

Knusel, B., Winslow, J. W., Rosenthal, A., Burton, L. E., Seid, D. P., Nikolics, K. and Hefti, F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc Natl Acad Sci U S A* 88: 961-965.

KO, L., Fu, W, Y., Ip, F. C., Ip, N, Y. (1998). Cloning and expression of a novel neurotrophin, Nt-7, from Carp. Mol. Cell. Neurosci. 11: 64-76

Kochhar, K.. S. and Iyer, A. P. (1996). Hepatocyte Growth Factor induces activation of Nck ad Nck and phospholipase C-gamma in lung carcinoma cells. Cancer lett 104: 163-169.

Koliatsos, V. E., Cayouette, M. H., Berkemeier, L. R., Clatterbuck, R. E., Price, D. L., and Rosenthal, A. (1994). Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons. Proc. Natl. Acad. Sci. U S A. 91:3304-3308.

Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H. and Price, D. L. (1993). Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* 10: 359-67.

Komada, M., Miyazawa, K., Ishii, T., and Kitamura, N. (1992). Characterization of hepatocyte growth factor receptors on Meth A cells. Eur. J. Biochem. 204: 857-64.

Komoda, M., and Kitamura, N. (1993). The cell dissociation and motility by scatter factor/hepatocyte growth factor are mediated through the cytoplasmic domain of the c-Met receptor. Oncogene 8: 2381-2390.

Korsching, S. and Thoenen, H. (1983a). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA* 80: 3513-3516.

Korsching, S., Auburger, G., Heumann, R., Scott, J. and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *Embo J* 4: 1389-93.

Korsching, S. and Thoenen, H. (1988). Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. *Devel. Biol.* 126: 40-46.

Korsmeyer, S. J., Shutte,r J. R., Veis, D. J., Merry, D. E., and Oltvai, Z. N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol. 4:327-332.

Kotzbauer, P. T., Lampe, P. A., Estus, S., Milbrandt, J. and Johnson, E. M. (1994). Postnatal development of survival responsiveness in rat sympathetic neurons to leukemia inhibitory factor and ciliary neurotrophic factor. *Neuron* 12: 763-773.

Kotzbauer, P. T., Lampe, P. A., Heuckeroth, R. O., Golden, J. P., Creedon, D. J., Johnson, E. M. Jr., and Milbrandt, J. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384:467-470.

Krasnoselsky, A., Massay, M. J., DeFrances, M., Michalopoulos, G., Zarnegar, R., and Ratner, N. (1994). Hepatocyte Growth Factor is a mitogen for schwann cells and is present in neurofibromas. J. Neurosci 12: 7284-7290.

Krebs, J. F., Armstrong, R. C., Srinivasan, A., Aja, T., Wong, A. M., Aboy, A., Sayers, R., Pham, B., Vu, T., Hoang, K., Karanewsky, D. S., Leist, C., Schmitz, A., Wu, J. C., Tomaselli, K. J, and Fritz L. C. (1999). Activation of membrane-associated procaspase-3 is regulated by Bcl-2. J Cell Biol. 144:915-926

Kushima, Y., Hama, T. and Hatanaka, H. (1992). Interleukin-6 as a neurotrophic factor for promoting the survival of cultured catecholaminergic

neurons in a chemically defined medium from fetal and postnatal rat. *Neurosci Res* 13: 267-280.

Lai, K. O., Fu, W. Y., Ip, F. C., Ip, N. Y. (1998). Cloning and expression of a novel neurotrophin, NT-7, from carp. Mol Cell Neurosci. 11:64-76.

Lam, A., Fuller, F., Miller, J., Kloss, J., Manthorpe, M., Varon, S. and Cordell, B. (1991). Sequence and structural organization of the human gene encoding ciliary neurotrophic factor. *Gene* 102: 271-6.

Lamballe, F., Klein, R. and Barbacid, M. (1991). The trk family of oncogenes and neurotrophin receptors. *Princess Takamatsu Symp* 22: 153-170.

Lamballe, F., Smeyne, R. J. and Barbacid, M. (1994). Developmental expression of trkC, the neurotrophin-3 receptor, in the mammalian nervous system. *J. Neurosci.* 14: 14-28.

Lamballe, F., Tapley, P., and Barbacid, M. (1993) trkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. EMBO J. 12:3083-3094.

Landmesser, L., and Pilar, G. (1974). Synaptic transmission and cell death during normal ganglionic development. *J. Physiol. (Lond)* 241: 737-749.

Large, T. H., Weskamp, G., Helder, J. C., Radeke, M. J., Misko, T. P., Shooter, E. M. and Reichardt, L. F. (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. *Neuron* 2: 1123-1134.

Larkfors, L., Lindsay, R. M. and Alderson, R. F. (1994). Ciliary neurotrophic factor enhances the survival of Purkinje cells in vitro. *Eur J Neurosci* 6: 1015-1025.

Le Douarin, N. (1986). Cell line segregation during peripheral nervous system ontogeny. *Science* 231: 1515-1522.

Lee, K. F., Davies, A. M. and Jaenisch, R. (1994). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* 120: 1027-1033.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y. A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* 341: 149-152.

Leingartner, A. and Lindholm, D. (1994). Two promoters direct the expression of the mouse NT-3 gene. *Eur. J. Neurosci.* 6: 1149-1159.

Lentz, S. I., Knudson, C. M., Korsmeyer, S. J., and Snider, W. D. (1999). Neurotrophins support the development of diverse sensory axon morphologies. J Neurosci 19:1038-1048.

Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P. and Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* 8: 1045-1053.

Levi-Montalcini, R., Caramia, F., Luse, S. A., and Angeletti, P. U.(1968). In vitro effects of the nerve growth factor on the fine structure of the sensory nerve cells. Brain Res. 2:347-362.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*: 1154-1162.

Lewin, G.R., and Barde, Y.-A. 1996. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19**: 289-317.

Lewis, J., Chevallier, A., Kieny, M., and Wolpert, L.. (1981). Muscle nerve branches do not develop in chick wings devoid of muscle. *L. Embryol. exp. Morph.* **64**: 211-232.

Li, F., Srinivasan, A., Wang, Y., Armstrong, R.C., Tomaselli, K. J., and Fritz, L. C. (1997a). Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. J Biol Chem. 272:30299-302305

Li, H., Bergeron, L., Cryns, V., Pasternack, M. S., Zhu, H., Shi, L., Greenberg, A., and Yuan, J. (1997b). Activation of caspase-2 in apoptosis. J Biol Chem. 272:21010-1017

Li, L., Oppenheim, R. W., Lei, M. and Houenou, L. J. (1994). Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. *J Neurobiol* 25: 759-66.

Li, M., Sendtner, M. and Smith, A. (1995). Essential function of LIF receptor in motor neurons. *Nature* 378: 724-727.

Li, R. and Bernd, P.(1992). Neurotrophin-3 increases neurite outgrowth and apoptosis in explants of the chicken neural plate. Dev Neurosci. 21:12-21.

Lin, E. Y., Orlofsky, A, Berger, M. S., and Prystowsky, M. B. (1993). Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. J Immunol. 151:1979-1988.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F.(1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 260:1130-1132.

Lin,C-H., Thompson, C. A., and Forscher, P. (1994). Cytoskeletal reorganization underlying growth cone motility. Curr.Opin. Neurobiol. 4: 640-647.

Lindsay, R. M., Thoenen, H. and Barde, Y. A. (1985). Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Devel. Biol.* 112: 319-328.

Lindsay, R.M. (1988). Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci.7:2394-2405.

Linette, G. P., and Korsmeyer, S. J.(1994). Differentiation and cell death: lessons from the immune system. Curr. Opin. Cell. Biol. 6:809-815.

Lipke, J. A., Gu, Y., Sarnecki, C., Caron, P. R., and Su, MS-S.(1996). Identification and characterization of CPP32/Mch2 homolog 1, a novel cysteine protease similar to CPP32. J. Biol. Chem. 271: 1825-1828.

Liu, X., Ernfors, P., Wu, H. and Jaenisch, R. (1995). Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature* 375: 238-241.

Liu, Y., Beedle, A .B., Lin, L., Bell, A. W., and Zarnegar, R. (1994). Identification of a cell-type specific transcriptional repressor in the promoter region of the mouse hepatocyte growth factor gene. Mol. Cell Biol. 14: 7046-7058.

Liu, Y., Tolbert, E. M., Sun, A. M., and Dworkin, L. D. (1996a). Primary structure of rat HGF receptor and induced expression in glomerular mesangial cells. Am. J. Physiol. 271: F679-F688.

Liu, Y., Tolbert, E. M., Sun, A.M., and Dworkin, L. D. (1996b). In vivo and in vitro evidence for increased expression of HGF receptor in kidney of diabetic rat. Am. J. Physiol. 271: F1202-F1210.

Liu, Y. (1998). The human hepatocyte growth factor receptor gene: complete structural organization and promoter characterization. Gene 215: 159-169.

Lockshin, R. A., and Beaulaton, J. (1981) Cell death: questions for histochemists concerning the causes of the various cytological changes. Histochem J. 13:659-666.

Loetscher, H., Schlaeger, E. J., Lahm, H. W., Pan, Y. C., Lesslauer, W., and Brockhaus, M. (1990). Purification and partial amino acid sequence analysis of two distinct tumor necrosis factor receptors from HL60 cells. J Biol Chem. 265:20131-20138.

Lokker, N. A., Mark, M. R., Luis, E. A., Bennet, G. L., Robbins, K. A., Baker, J. B., and Godowski, P J. (1992). Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. EMBO. J. 11: 2503-2510.

Longati,P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994). Tyrosine 1234-1235 are critical for activation of the tyrosine kinase encoded by the Met proto-oncogene (HGF) receptor. Oncogene 1: 49-57.

Lou, L., Yan, L. Y., and Jan, Y. N. (1997). Rho family small GTP-binding proteins in growth cone signalling. Curr.Opin. Neuro. Biol. 7: 81-86.

Lu, Q. L., Poulsom, R., Wong, L., and Hanby, A. M. (1993). Bcl-2 expression in adult and embryonic non-haematopoietictissues. J Pathol. 169:431-437.

Maggiora, P., Gambarotta, G., Olivero, M., Giordano, S., Di Renzo, M. F. and Comoglio, P. M. (1997). Control of invasive growth by the HGF receptor family. *J. Cell Physiol.* 173: 183-186.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P. M., Klein, R., and Ponzetto, C. (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell *87*: 531-542.

Maina, F., Hilton, M. C., Ponzetto, C., Davies, A. M., and Klein, R. (1997). Met receptor signalling is required for sensory nerve development. Genes Dev. *11*: 3341-3350.

Maina, F., Hilton, M. C., Andres, R., Wyatt, S., Klein, R. and Davies, A .M. (1998). Multiple roles for hepatocyte growth factor in sympathetic neuron development. *Neuron* 20: 835-846.

Maisonpierre, P. C., Belluscio, L., Squinto, S., Ip, N. Y., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990a). Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* 247: 1446-1451.

Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990b). NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* 5: 501-509.

Maisonpierre, P. C., Le Beau, M. M., Espinosa, R 3d., Ip, N. Y., Belluscio, L, de la Monte, S. M., Squinto, S., Furth, M. E., and Yancopoulos, G. D. (1991). Human and rat brain-derived neurotrophic factor andneurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics.10: 558-568.

Mallett, S., Fossum, S., and Barclay, A. N. (1990). Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes--a molecule related to nerve growth factor receptor. EMBO J . 9:1063-1068.

Manthorpe, M., Davis, G. E., and Varon, S. (1985). Purified proteins acting on cultured chick embryo ciliary ganglion neurons. Fed. Proc. 44:2753-2759.

Manthorpe, M., Skaper, S. D., Williams, L. R. and Varon, S. (1986). Purification of adult rat sciatic nerve ciliary neuronotrophic factor. *Brain Res* 367: 282-6.

Martinou, J. C., et al. (1994). Overexpression of bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischaemia. *Neuron* . 13:1017-1030.

Martinou, J. C., Martinou, I. and Kato, A. C. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. *Neuron* 8: 737-744.

Martinou, I., Missotten, M., Fernandez, P. A., Sadoul, R., and Martinou, J. C. (1998). Bax and Bak proteins require caspase activity to trigger apoptosis in sympathetic neurons. Neuroreport. 9:15-19

Martin-Zanca, D., Mitra, G., Long, L. K. and Barbacid, M. (1986). Molecular characterization of the human *trk* oncogene. 51: 983-992.

Martin-Zanca, D., Oskam, R., Mitra, C., Copeland, T. and Barbacid, M. (1989). Molecular and biochemical characterisation of the human *trk* proto-oncogene. *Mol. Cell. Biol.* 9: 24-33.

Martin-Zanca, D., Barbacid, M. and Parada, L. F. (1990). Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev* 4: 683-94.

Massague, J., Attisano, L., and Wrana, J.L. (1994). The TGF-Family and its composite receptors. Trends in Cell Biology 4: 172-177.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G. and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* 365: 27-32.

Matsumoto, K., Kagoshima, M., and Nakamura, T. (1995). Hepatocyte growth factor as a potent survival factor for rat pheochromocytoma PC12 cells. Exp. Cell. Res. 220: 71-78.

McAllister, A. K., D.C., L. and Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15: 791-803.

McAllister, A. K., Katz, L. C., and Lo, D. C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. Neuron. 17:1057-1064

McAllister, A. K, Katz, L. C., and Lo, D. C. (1997). Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron. 18:767-778

McDonald, N. Q., Lapatto, R., Murray, R. J., Gunning, J., Wlodawer, A. and Blundell, T. L. (1991). New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. *Nature* 354: 411-414.

McDonald, N. Q., Panayotatos, N., and Hendrickson, W. A. (1995). Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. EMBO J. 14:2689-2699

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn J. P., and Korsmeyer, S. J. (1989). Bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57:79-88.

McDonnell, T. J., Nunez, G., Platt, F.M., Hockenberry, D., London, L., McKearn, J. P., and Korsmeyer, S.J. (1990). Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol. Cell. Biol.* 10: 1901-1907.

Meakin, S. O. and Shooter, E. M. (1992). The nerve growth factor family of receptors. *Trends Neurosci* 15: 323-31.

Meier, R., Becker, A. M., Gotz, R., Heumann, R., Shaw, A. and Thoenen, H. (1986). Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. *Embo J.* 5: 1489-93.

Merry, D.E., Veis, D. J., Hickey, W.F., and Korsmeyer, S. J. (1994). bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS *Development.* 120:301-311.

Michaelidis, T. M., Sendtner, M., Cooper, J. D., Airaksinen, M. S., Holtmann, B., Meyer, M., and Thoenen, H. (1996). Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic neurons and

sensory neurons during the early postnatal development. *Neuron* 17:75-89.

Michalopoulos, G., Houck, K. A., Dolan, M. L., and Leutteke, N. C. (1984). Control of hepatocyte replication by two serum factors. Cancer Res. 44: 4414-4419.

Middlemas, D. S., Lindberg, R. A. and Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol Cell Biol* 11: 143-53.

Middleton, G., Pinon, L., Wyatt, S., and Davies, A. M. (1998). Bcl-2 Accelerates the Maturation of Early Sensory Neurons. J Neurosci. 18:3344-3350.

Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M, G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S., Golden, J. P., Davies, J. A., Vejsada, R., Kato, A. C., Hynes, M., Sherman, D., Nishimura, M., Wang, L. C., Vandlen, R., Moffat, B., Klein, R. D., Poulsen, K., Gray, C., Garces, A., Johnson, E. M. Jr., et al. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20:245-253

Miller, T. M., Moulder, K. L., Knudson, C. M., Creedon. D. J., Deshmukh. M., Korsmeyer, S. J., and Johnson, E. M. Jr. (1997). Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol.139:205-217

Minichiello, L., F., P., Vazquez, E., Schimmang, T., Hokfelt, T., Represa, J. and Klein, R. (1995). Differential effects of combined trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. *Development* 121: 4067-4075.

Minn, A. J., Boise, L. H., and Thompson, C. B. (1996). Bcl-x(S) anatagonizes the protective effects of Bcl-x(L). J Biol Chem. 271:6306-6312.

Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., Thompson, C. B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature. 385:353-357.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A., and Yaun, J. (1993). Induction of apoptosis in fibroblasts by IL-1b converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 75: 653-660.

Miyazaki, K., Kitamura, A., Naka, D., and Kitamura, N. (1991). An alternatively processed mRNA generated from human hepatocyte growth factor gene. Euro. J. Biochem. 197: 15-22.

Miyazaki, K., Tsubouchi, H., Naka, D., Takahashi, M., Okigaki, N., Arakaki, H., Nakayama, S., Hirono, O., Sakiyama, K., Takahashi, E., Gohda, E., Daikuhara, Y., and Kitamura, N. (1989). Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem. Biophys. Res. Comm. 163: 967-973.

Miyazaki, M., Gohda, E., Kaji, K., and Namba, M.(1998). Increased Hepatocyte Growth Factor production by aging human fibroblasts mainly due to autocrine stimulation by Interleukin-1. Biochem. Biophys. Re. Comm. 246: 255-260.

Miyazawa, K., Kitamura, A., and Kitamura, N. (1991). Structural organization and the transcription initiation site of the human hepatocyte growth factor gene. Biochemistry. 38: 9170-9176.

Mizuno, K., Takehara, T., and Nakamura, T. (1992). Proteolytic activation of a single-chain precursor of hepatocyte growth factor by extracellular serine-protease. Biochem. Biophys. Res. Comm. 189: 1631-1638.

Montesano,R., Matsumoto, K., Nakamura, T., and Orci, L.(1991). Identification of a fibroblast-derived epithelial morphogen as Hepatocyte Growth Factor. Cell 67: 901-908.

Moody, S. A., Quigg, M. S., and Little, C. D. (1989). Extracellular matrix components of the peripheral pathway of chick trigeminal axons. J. Comp. Neur. 283: 38-53.

Moore, J. B., Mobley, W. C., and Shooter, E. M. (1974). Proteolytic modification of beta nerve growth factor protein. Biochemistry. 13: 833-840.

Moriyama, T., Kataoke, H., Tsubouchi, H., and Koono, M.(1995). Concomitant expression of hepatocyte growth factor (HGF), HGF activator and c-met genes in human glioma cells in vitro. FEBS lett. 372: 78-82.
motility. *J. Biol. Chem.* 271: 14119-14123

Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature. 381:335-341.

Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., and Molineaux, S. M. (1995). Molecular cloning and pro-apoptotic activity of ICErel II and ICErel III, member of the ICE/CED-3 family of cysteine proteases J. Bio. Chem. 270: 15870-15876.

Murphy, M., Reid, K., Brown, M. A. and Bartlett, P. F. (1993). Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* 117: 1173-1182.

Muzio, M., Chinnaiyan, A, M., Kischkel, F, C., O' Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., and Gentz, R. (1996). Flice, a novel FADD-homologous ICE/CED-3 like protease, is recruited to the CD95 (fAS/apo-1) death-inducing signalling complex. Cell. 85: 817-827.

Nagaike, M., Hirao, S., Tajima, H., Noji, S., Taniguchi, S., Matsumoto, K., and Nakamura, T. (1991). Renotropic functions of hepatocyte growth factor in renal regeneration after unilateral nephrectomy. J. Biol. Chem. 266: 22781-22784.

Nakamura, T., Nawa, K., and Ichihara, A. (1984). Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem. Biophys. Res. Comm. 122: 1450-1459.

Nakamura, T., Teramoto, H., and Ichihara, A. (1986).Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. Proc. Natl. Acad. Sci. USA 83: 648-6493.

Nakamura, T., Nawa, K., Ichihara, A., Kaise, N., and Nishino, T. (1987). Purification and subunit structures of hepatocyte growth factor from rat platelets. FEBS Letts. 224: 311-316.

Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989). Molecular cloning and expression of human hepatocyte growth factor. Nature 342: 440-443.

Nakayama, K., Negishi, I., Kuida, K., Shinkai, Y., Louie, M, C., Fields, L. E., Lucas, P. J., Stewart, V., Alt, F,W. (1993). Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. *Science* 261:1584-1588.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D. Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice:occurrence of gray hair, polycystic kidney disease, and lymphocytopenia *Proc. Natl. Acad. Sci. USA.* 91:3700-3704.

Naldini, L., Weidner, M. K., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R. P., Hartmann, G., Zarnegar, R., Michalopoulos, G. K., Birchmeier, W., and Comoglio, P. M. (1991a). Scatter factor and hepatocyte growth factor are indistinguishable ligands for the Met receptor. EMBO. J. 10: 2867-2878.

Naldini, L., Vigna, E., Narsimhan, R. P., Gaudino, G., Zarnegar, R., Michalopoulos, G.K., and Comoglio, P.M. (1991b). Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-Met. Oncogene 4: 501-504.

Naldini, L., Vigna, E., Ferracini, P., Longati, L., Gandino, G., Prat, M., and Comoglio, P. M. (1991c). The tyrosine kinase encoded by the Met proto-oncogene is activated by autophosphorylation. Mol. Cell. Biol. 11: 1793-1803.

Naldini, L., Vigna, E., Bardelli, A., Follenzi, A., Galimi, F., and Comoglio, P.M. (1995). Biological activation of pro-HGF (Hepatocyte Growth Factor) by urokinase is controlled by a stoichiometric reaction. J. Biol. Chem. 270: 603-611.

Naveilhan, P., Baudet, C., Mikaels, A., Shen, L., Westphal, H., and Ernfors, P. (1998). Expression and regulation of GFRalpha3, a glial cell line-derived neurotrophic factor family receptor.Proc. Natl. Acad. Sci. U S A. 95:1295-1300

Negrini, M., Silini, E., Kozak, C., Tsujimoto, Y., and Croce, C. M. (1987). Molecular analysis of mbcl-2: structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma. Cell. 49:455-463.

Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J Biol Chem. 268:25265-25268.

Nguyen, L., Holgado-Madruga, M., Maroun, C., Fixman, E .D. Kamikura, D., Fournier, T., Charest, A., Tremblay, M. L., Wong, A. J., and Park, M. (1997). Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional Grb2 binding site involving tyrosine 1356. J. Biol. Chem. 272: 20811-20819.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P. Ding, C. K., and Gallant, M. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature, 376: 37-43.

Nilsson, A. S., Fainzilber, M., Falck, P., and Ibanez CF. (1998). Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. FEBS Lett. 424:285-90.

Ninkina, N., Adu, J., Fischer, A., Pinon, L., Buchman, V. and Davies, A. M. (1996). Expression and function of TrkB variants in developing trigeminal neurons. *EMBO J.* 15: 6385-6393.

Ninkina, N., Grashchuck, M., Buchman, V. L. and Davies, A. M. (1997). TrkB variants with deletions in the leucine-rich motifs of the extracellular domain. *JBC* 272: 13019-13025.

Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. (1995). HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development 121: 2897-2908.

Nishi, R. (1994). Neurotrophic Factors: two are better than one. Science 265: 1052-1053.

Nomoto, S., Ito, S., Yang, L .X., and Kiuchi, K. (1998). Molecular cloning and expression analysis of GFR alpha-3, a novel cDNA related to GDNFR alpha and NTNR alpha.Biochem Biophys Res Commun. 244: 849-853.

Nunez. G., London, L., Hockenbery, D., Alexander, M., McKearn, J. P., and Korsmeyer, S. J. (1990). Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol. 144:3602-3610.

Nunez, G., Clarke, M. F. (1994). The Bcl-2 family of proteins: regulators of cell death and survival. TRENDS IN CELL BIOLOGY, 4: 399-403.

Oakley, R . A., and Tosney, K. W. (1993). Contract-mediated mechanisms of motor axon segmentation J.Neurosci. 13: 3773-3792.

Oakley, R. A., Garner, A. S., Large, T. H. and Frank, E. (1995). Muscle sensory neurons require neurotrophin-3 from peripheral tissues during the period of normal cell death. *Development* 121: 1341-1350.

Ockel, M., Lewin, G. R. and Barde, Y. A. (1996). In vivo effects of neurotrophin-3 during sensory neurogenesis. *Development* 122: 301-307.

O'Connor, L., Strasser, A., O'Reilly, L. A., Hausmann, G., Adams, J. M., Cory, S., and Huang, D. C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 17:384-395.

O'Connor, T. P., and Bentley, D. (1993). Accumulation of actin in subsets of pioneer growth cone filopodia in response to neuronal epithelial guidance cues in situ. J.Cell. Biol. 123, 4: 935-948.

Oda, T., Ohta, M., Inoue, S., Ikeda, K., Furukawa, S., and Hayashi, K. (1989). Amino acid sequence of nerve growth factor purified from the venom of the Formosan cobra Naja naja atra. Biochem Int. 4: 909-917.

Oh, Y.J., Swarzenski, B. C., and O'Malley, K. L. (1996). Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth. *Neurosci Lett.* 202:161-164.

Ohsawa, F., Widmer, H. R., Knusel, B., Denton, T. L. and Hefti, F. (1993). Response of embryonic rat hippocampal neurons in culture to neurotrophin-3, brain-derived neurotrophic factor and basic fibroblast growth factor. *Neuroscience* 57: 67-77.

Okajima, A., Miyazawa, K., and Kitamura, N. (1990). Primary structure of rat hepatocyte growth factor and induction of its mRNA during liver regeneration following hepatic injury. Eur. J. Biochem. 193: 375-381.

Okigaki, M., Komada, M., Uehara, Y., Miyazawa, K., and Kitamura, N. (1992). Functional characterization of human hepatocyte growth factor mutants obtained by deletion of structural domains. Biochemistry 31: 9555-9561.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 74:609-619.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Ann. Rev. Neurosci.* 14: 453-501.

Oppenheim, R. W., Prevette, D., Yin, Q. W., Collins, F. and MacDonald, J. (1991). Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. *Science* 251: 1616-1618.

Oppenheim, R. W., Qin-Wei, Y., Prevette, D. and Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature* 360: 755-757.

Oppenheim, R.W. (1996). Neurotrophic survival molecules for motoneurons: an embarrassment of riches. *Neuron* 17: 195-197.

Palmatier, M. A., Hartman, B. K., and Johnson, E. M Jr. (1984). Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons.J Neurosci. 4:751-756.

Pan, G., O'Rourke, K., and Dixit, V. M. (1998). Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol Chem. 273:5841-5845

Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S .J. Blair, D. G., and Vande woude, G.F. (1986). Mechanism of met oncogene activation. Cell 45: 895-904.

Park, M., Dean, M., Kaul, K., Braun, M.J. Gonda, M. A., and Vande-Woude, G.(1987). Sequence of Met protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc. Natl. Acad. Sci. USA 84: 6379-6383.

Paul, G., and Davies, A. M. (1995). Trigeminal sensory neurons require extrinsic signals to switch neurotrophin dependence during the early stages of target field innervation. *Devel. Biol.* 1995: 171: 590-605.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S., Darbonne, W. C., Knutzon, D. S., Yen, R., Chien, K. R., Barker, J. B. and Wood, W. I. (1995a). Expression cloning of cardiotrophin-1, a cytokine that induces cardiac myocyte hypertrophy. *Proc. Natl. Acad. Sci. USA* 92: 1142-1146.

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. and Wood, W. I. (1995b). Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J. Biol. Chem.* 270: 10915-10922.

Pennica, D., Arce, V., Wsanson, T. A., Vejsada, R., Pollock, R. A., Armanini, M., Dudley, K., Phillips, H. S., Rosenthal, A., Kato, A. C. and Henderson, C. E. (1996). Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron* 17: 63-74.

Phillips, H. S., Hains, J. M., Laramee, G. R., Rosenthal, A. and Winslow, J. W. (1990). Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. *Science* 250: 290-294.

Pincus, D. W., Di, C. B. E., and Black, I. B. (1990). Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. Nature *343*: 564-7.

Pinon, L G. P, Minichiello, L., Klein, R., Davies, A. M (1996). Timing of neuronal beath in TrkA, TrkB and TrkC mutant embryos reveals developmental changes in sensory neuron dependence on Trk signalling. Development 122: 3255-3261.

Pinon, L. G., Middleton, G., and Davies, A. M. (1997). Bcl-2 is required for cranial sensory neuron survival at defined stages of embryonic development. Development 124: 4173-4178

Pirvola, U., Ylikoski, J., Palgi, J., Lehtonen, E., Arumae, U. and Saarma, M. (1992). Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc. Natl. Acad. Sci. U S A.* 89: 9915-9919.

Ponzetto, C., Giordano, S., Peverali, F., Della Valle, G., Abata, M. L., Vaula, G., Comoglio, P. M. (1991). C-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. Oncogene 6: 553-559.

Ponzetto, C., Zhen, Z., Audero, E., Maina, F., Bardelli, A., Basile, M. Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M. D., and Comoglio, P. M. (1993). A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor / scatter factor. Mol. Cell. Biol. 13: 4600-4608.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell *77*, 261-271.

Ponzetto, C., Zhen, Z., Audero, E., Maina, F., Bardelli, A., Basile, M. L., Giordano, S., Narsimhan, R., and Comoglio, P. M. (1996). Specific coupling of GRB2 from the Met receptor: differential effect on transformation and motility. J. Biol. Chem. 271: 14119-14123.

Prat, M., Crepaldi, T., Gandino, L., Giordano, S., Longati, P., and Comoglio, P. M. (1991). C-terminal truncated form of the Met, the Hepatocyte Growth Factor Receptor. Mol. Cell. Biol. 11: 5954-5962.

Purves, D., Snider, W. D. and Voyvodic, J. T. (1988). Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* 336: 123-128.

Puschel, A.W., Adams, R. H., and Betz, H. (1996). The sensory Innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. *Mol. Cell. Neurosci.* 7: 419-431.

Qin, H., Srinivasula, S. M., Wu, G., Fernandes-Alnemri, T., Alnemri, E.S., and Shi, Y. (1999). Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1. Nature. 399:549-557

Rabizadeh, S., Oh, J., Zhong, L. T., Yang, J., Bitler, C. M., Butcher, L. L. and Bredesen, D. E. (1993). Induction of apoptosis by the low-affinity NGF receptor. *Science* 261: 345-348.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. and Shooter, E. M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* 325: 593-597.

Radziejewski, C., Robinson, R. C., Di, S. P. and Taylor, J. W. (1992). Dimeric structure and conformational stability of brain-derived neurotrophic factor and neurotrophin-3. *Biochemistry* 31: 4431-6.

Raffioni, S., Bradshaw, R. A., and Buxser, S. E. (1993). The receptors for nerve growth factor and other neurotrophins. Annu Rev Biochem. 62:823-850.

Ratts, V. S., Flaws, J. A., Kolp, R., Sorenson, C. M., and Tilly, J. L.(1995). Ablation of bcl-2 gene expression decreases the numbers ofoocytes and primordial follicles established in the post-natal female mouse gonad. Endocrinology. 136:3665-3668.

Reynolds, J. E, Yang, T., Qian, L., Jenkinson, J. D., Zhou, P., Eastman, A., and Craig, R. W. (1994). Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer Res. 54: 6348-6352.

Riddle, D. R., Lo, D. C. and Katz, L. C. (1995). NT-4 mediated rescue of lateral geniculate neurons from effects of monocular deprivation. *Nature* 9: 189-191.

Ringstedt, T., Lagercrantz, H., and Persson, H. (1993). Expression of members of the trk family in the developing postnatal rat brain. Brain Res Dev Brain Res. 72:119-131.

Robinson, M., Buj-Bello, A., and Davies, A. M. (1996a). Paracrine interactions of BDNF involving NGF-dependent embryonic sensory neurons. Mol Cell Neurosci 7:143-151.

Robinson, M., Adu, J., and Davies, A. M. (1996b). Timing and regulation of trkB and BDNF mRNA expression in placode-derived sensory neurons and their targets. Eur J Neurosci. 8:2399-2406.

Robinson, R. C., Grey, L. M., Staunton, D., Vankelecom, H., Vernallis, A. B., Moreau, J. F., Stuart, D. I., Heath, J. K., and Jones, E. Y. (1994). The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. Cell. 77:1101-1116.

Rodrigues, G. A., Naujokas, MA., and Park, M. (1991). Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. Mol. Cell. Biol. 11: 2962-2970.

Rodrigues, G. A., and Park, M. (1993). Dimerization mediated through a leucine zipper activates the oncogene potential of the met receptor tyrosine kinase. Mol. Cell. Biol. 13: 6711-6722.

Rodrigues G, A., Park, M., Schlessinger, J. (1997). Activation of the JNK pathway is essential for transformation by the Met oncogene. EMBO J. 16: 2634-2645.

Rodriguez-Tebar, A., Dechant, G., Gotz, R. and Barde, Y. A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* 11: 917-922.

Rogers, L. A., and Cowen, W. M. (1974). The development of the mesencephalic nucleus of the trigeminal nerve in the chick. *J. Comp. Neurol.* 147:

291-320.

Rohrer, H., and Thoenen, H. (1987). Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. J. Neurosci. *7*: 3739-3748.

Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., and Vande Woude, G. F. (1992). Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. Mol. Cell. Biol. 11: 5152-5158.

Rong, S., Segal, S., Anver, M., Resau, J.H., and Vande-Woude, G.F. (1994). Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/ scatter factor autocrine stimulation. Proc. Natl. Acad. Sci. USA, 91: 4731-4735.

Rosen, E. M., Meromsky, L., Setter, E., Vinter, D.W., and Goldberg, I. D. (1990). Purified scatter factor stimulates epithelial and vascular endothelial cell migration. Proc. Soc. Exp. Biol. Med. 195: 34-43.

Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K. and Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4: 767-773.

Rosse, T., Olivier, R., Monney, L., Rage,r M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998). Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature. 391:496-469

Rothman, T. P., Gershon, M. D., and Holtzer, H. (1978). The relationship of cell division to the acquistion of adrenergic characteristics by developing sympathetic ganglion cell precursors. Devel. Biol. *65*: 322-341.

Rothman, T. P., Speccht, L. A., Gershon, M. D., John, T. H., Teitelman, G., Pickel, V. M., and Reis, D. J. (1980). Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. Proc. Natl. Acad. Sci. USA *77*: 6221-6225.

Rubin, J. S., Chan, A,M.L., Bottaro, D, P., Burgess, W. H., Taylor, W. G., Cech, A. C., Hirschfield, D.W., Wong, J., Miki, T., Finch, P. W., and Aaronson, S. A.(1991). A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc. Natl. Acad. Sci. USA, 88:415-419.

Ruit, K. G., Osborne, P.A., Schmidt, R.E., Johnson, E.M Jr., and Snider, W.D. (1990) Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. J Neurosci. 10: 2412-2419.

Ruit, K. G., and Snider, W. D. (1991). Administration or deprivation of nerve growth factor during development permanently alters neuronal geometry. J Comp Neurol.314 :106-113.

Ruit, K. G., Elliott, J. L., Osborne, P. A., Yan, Q., and Snider, W. D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron 18:573-587.

Ryden, M., Murray-Rust, J., Glass, D., Ilag, L. L., Trupp, M., Yancopoulos, G. D., McDonald, N. Q., and Ibanez, C. F. (1995). Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signalling. EMBO J. 14:1979-1990.

Ryden, M., Hempstead, B., and Ibanez, C. F. (1997). Differential modulation of neuron survival during development by nerve growth factor binding to the p75 neurotrophin receptor. J Biol Chem. 272:16322-16328.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R. B., Cate, R. L. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc. Natl. Acad. Sci. U S A. 94:6238-6243.

Sawai, H., Clarke, D. B., Kittlerova, P., Bray, G. M. and Aguayo, A. J. (1996). Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from regenerating retinal ganglion cells. *J. Neurosci* 16: 3887-3894.

Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H., Gatanaga, T., Granger, G. A., Lentz, R., and Raab, H. (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell. 61:361-370.

Schecterson, L. C. and Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron* 9: 449-463.

Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M., and Reed, J. C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U S A. 94:5113-5118.

Schimmang, T., Minichiello, L., Vazquez, E., Joac, I. S., Giraldez, F., Klein, R. and Represa, J. (1995). Developing inner ear sensory neurons require TrkB and TrkC receptors for innervation of their peripheral targets. *Development* 121: 3381-3391.

Schlesinger, P. H., Gross, A., Yin, X. M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S. J. (1997). Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc. Natl. Acad. Sci. U S A. 94:11357-11362.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. Nature *373*: 699-702.

Schnell, L., Schneider, R., Kolbeck, R., Barde, Y. A., and Schwab, M. E. (1994). Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature.* 367: 170-173.

Schober, A., Wolf, N., Huber, K., Hertel, R., Krieglstein, K., Minichiello, L., Kahane, N., Widenfalk, J., Kalcheim, C., Olson, L., Klein, R., Lewin, G. R., and Unsicker, K. (1998). TrkB and neurotrophin-4 are important for development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla. J Neurosci 18: 7272-7284.

Schoenwolf, G. C., and Smith, J. L. (1990). Mechanisms of neurulation: traditional viewpoint and recent advances. Development 109:243-270.

Schwarz, M. A., Fisher, D., Bradshaw, R. A. and Isackson, P. J. (1989). Isolation and sequence of a cDNA clone of beta-nerve growth factor from the guinea pig prostate gland. *J Neurochem* 52: 1203-1299.

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I. and Rutter, W. J. (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* 302: 538-540.

Scott, S. A., and Davies, A. M. (1990). Inhibition of protein synthesis prevents cell death in sensory and parasympathetic neurons deprived of neurotrophic factor in vitro. J Neurobiol. 21:630-638.

Seki, T. I., Ihara, A., Sugimara, M., Shimonishi, T., Nihizawa, O., Asami, M., Hagiya, T., Nakamura, T., and Shimizu, S. (1990). Isolation and expression of cDNA for different form of hepatocyte growth factor from human leukocyte. Biochem. Biophys. Res. Comm. 172: 321-327.

Selby, M. J., Edwards, R., Sharp, F. and Rutter, W. J. (1987). Mouse nerve growth factor gene: structure and expression. *Mol Cell Biol* 7: 3057-3064.

Seldon, W.A. (1986). Simplifying cardiac rehabilitation. Med. J. Aust.8: 144-395.

Sendtner, M., Kreutzberg, G. W. and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* 345: 440-441.

Sendtner, M., Stockli, K. A. and Thoenen, H. (1992b). Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *J Cell Biol* **118**, 139-48.

Sendtner, M., Carroll, P., Holtmann, B., Hughes, R. A. and Thoenen, H. (1994). Ciliary neurotrophic factor. *J. Neurobiol.* 25: 1436-1453.

Sendtner, M., Gotz, R., Holtmann, B., Escary, J. L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P., and Thoenen, H. (1996) Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr Biol. 6:686-694

Serafini, T., Kennedy, T. E., Galko., Mirzayan, C., Jessell, T. M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C.elegans UNC-6. Cell 78: 409-424.

Seto, M., Jaege, U., Hockett, R. D., Graninger, W., Bennett, S., Goldman, P., and Korsmeyer, S. J. (1988). Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J. 7:123-131.

Shelton, D. L. and Reichardt, L. F. (1984). Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc Natl. Acad. Sci. USA.* 81: 7951-7915.

Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A., and Higashio, K. (1991). Tumor cytotoxic factor/hepatocyte growth factor from human fibroblast: cloning of its cDNA, purification and characterization of recombinant protein. Biochem. Biophys. Res. Comm. 180: 1151-1158.

Shimizu. S., Narita, M., and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature. 399:483-487

Shintani, A., Ono, Y., Kaisho, Y., Sasada, R. and Igarashi, K. (1993). Identification of the functional regulatory region of the neurotrophin-3 gene promoter. *Brain Res Mol Brain Res* 17: 129-134.

Shiota, G., Rhoads, D. B., Wang, T.C., Nakamura, T., and Schmidt, E. V. (1992). Hepatocyte Growth Factor inhibits growth of hepatocellular carcinoma cells. Proc. Natl. Acad. Sci. USA, 89: 373-377.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A. and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature* 368: 246-249.

Smith, A. P., Greene, L. A., Fisk, H. R., Varon, S., and Shooter, E. M. (1969). Subunit equilibria of the 7S nerve growth factor protein. Biochemistry. 8: 4918-4926.

Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science. 248:1019-1023.

Smith, C. L. (1994). Cytoskeletal movements and substrate interactions during initiation of neurite outgrowth by sympathetic neurons in vitro. J. Neurosci.14: 384-398.

Smith, S. J. (1988). Neuronal cytomechanics: The actin-based motility of growth cones. Science 242 : 708-714.

Snider, W. D. (1988). Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. J Neurosci. 7:2628-2634

Sonnenberg, E., Meyer, D., Weidner, K. M., and Birchmeier, C. (1993). Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J. Cell Biol. *123*: 223-235.

Stahl, N. and Yancopoulos, G. D. (1994). The tripartite CNTF receptor complex: activation and signalling involves components shared with other cytokines. *J. Neurobiol.* 25: 1454-1466.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N., and Yancopoulos, G. D. (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* . 263: 92-95.

Stern , C., Ireland, G.W., Herrick, S. E. Gherardi, E., Gray, J., Perryman, M., and Stoker, M. (1990). Epithelial scatter factor and development of the chick embryonic axis. Development 110: 1271-1284.

Stockli, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D. and Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* 342: 920-923.

Stockli, K. A., Lillien, L. E., Naher-Noe, M., Breitfeld, G., Hughes, R. A., Raff, M. C., Thoenen, H., and Sendtner, M. (1991). Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in therat brain. J Cell Biol. 115:447-459.

Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987). Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 327: 239-242.

Stoker, A., and Dutta, R. (1998). Protein tyrosine phosphatases and neural development. BioEssays 20: 463-472.

Strasser, A., Harris, A. W., and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell. 67:889-899.

Streit, A., Stern, C. D., Thery, C., Ireland, G. W., Aparicio, S., Sharpe, M.J., and Gherardi, E. (1995). A role of HGF/SF in neural induction and its expression in Hensen's node during gastrulation. Development 121: 813-824.

Studer, L., Spenger, C., Seiler, R. W., Altar, C. A., Lindsay, R. M., and Hyman, C. (1995). Comparison of the effects of the neurotrophins on the morphological structure of dopaminergic neurons in cultures of rat substantia nigra. Eur J Neurosci. 7:223-233

Tabata, M.J., Kim, K., Liu, J.G., Ymashita, K., Matsumura, T., Kato, J., Iwamoto, M., Wakisaka, S., Matsumoto, K., Nakamura, T., Kumegawa, M., and Kurisu, K. (1996). Hepatocyte Growth Factor is involved in the morphogenesis of tooth germ in murine molars. Development 122: 1243-1251.

Tabebayashi, T., Iwamoto, M., Jikko, A., Matsumura, T., Enomoto-Iwamoto, M., Myouki, F., Koyama, E., Yamaai, T., Matsumoto, K., Nakamura, T., Kurisu, K., and Noji, S. (1995). Hepatocyte Growth Factor/Scatter factor modulates cell motility, proliferation, and proteoglycan synthesis of chondrocytes. J. Cell Biol. 129: 1411-1419.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirando, T., and Kishimoto, T. (1989). Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell, 58. 573-581

Tajima, H., Matsumoto, K., and Nakamura, T.(1991). Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines. FEBS letts. 291: 229-232

Takahashi, M., Ota, S., Ogura, K., Nakamura, T., and Omata, M. (1995). Hepatocyte Growth Factor stimulates wound repair of the rabbit esophageal epithelial cells in primary culture. Biochem. Biophys. Res. Comm. 216: 298-305.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J.C. (1995). Cloning and functional analysis of BAG-1: a novel bcl-2 binding protein with anti-cell death. Cell. 80: 279-284.

Takayama, H., La Rochelle, W. J., Anver, M., Bockman, D. E., and Merlin, G. (1996). Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. Proc. Natl. Acad. Sci. U S A.;93:5866-5871

Tanaka, S., Saito, K., and Reed, J. C. (1993). Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival. J Biol Chem. 268:10920-10926.

Tashiro, K., Hagiya, M., Nishizawa, T., Seki, T., Shimonishi, M., Shimizu, S., and Nakamura, T. (1990). Deduced primary structure of rat hepatocyte growth factor and expression of the mRNA in rat tissues. Proc. Natl. Acad. Sci. USA, 87: 3200-3204.

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., and Allen, R. E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Developmental Biology 194: 114-128.

Tempest, P. R., Stratton, M. R., and Cooper, C. S. (1988). Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. Br. J. Cancer. 58: 3-7.

Tewari, M., Quan, L. T., O'Rouke, K.., Desnoyers, S., Zeng, Z., and Beidler, D. R. (1995). Yamma/CPP32 beta, a mammalian homolog of CED-3, is a Crma-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell, 81: 801-809.

Thaler, C. D., Suhr, L., Ip, N. and Katz, D. M. (1994). Leukemia inhibitory factor and neurotrophins support overlapping populations of rat nodose sensory neurons in culture. *Dev Biol* 161: 338-344.

Theiler K., *The House Mouse (Development and normal stages from fertilisation to 4 weeks).* (Springer-Verlag, Berlin, 1972).
these factors for survival. Neuron *9*, 139-150.

Thewke, D. P. and Seeds, N. W. (1996). Expression of hepatocyte growth factor/scatter factor, its receptor, c- met, and tissue-type plasminogen activator during development of the murine olfactory system. *J. Neurosci.* 16: 6933-6944.

Thewke, D. P., and Seeds, N. W. (1999). The expression of mRNAs for hepatocyte growth factor/scatter factor, its receptor c-met, and one of its activators tissue-type plasminogen activator show a systematic relationship in the developing and adult cerebral cortex and hippocampus. *Brain Res.* 821: 356-367.

Thier, M., Hall, M., Heath, J. K., Pennica, D., and Weis, J. (1999). Trophic effects of cardiotrophin-1 and interleukin-11 on rat dorsal root ganglion neurons in vitro. Brain. Res. Mol. Brain. Res.64:80-84.

Thoenen, H. and Barde, Y. (1980). Physiology of nerve growth factor. *Physiol. Rev.* 60: 1284-1335.

Thoenen. (1991). The changing scene of neurotrophic factors. Trends Neurosci. 5:165-170.

Thompson, J., Doxakis, E., Pinon, L. G., Strachan, P., Buj-Bello, A., Wyatt, S., Buchman, V. L., and Davies, A. M. (1998). GFRalpha-4, a new GDNF family receptor.Mol Cell Neurosci. 11:117-126

Thomas, K. A., Silverman, R. C., Jeng, I., Baglan, N. C., and Bradshaw, R. A. (1981). Electrophoretic heterogeneity and polypeptide chain

structure of the gamma subunit of mouse submaxillary 7S nerve growth factor. J. Bilo. Chem. 256: 9147-9155.

Thomson, T. M., Rettig, W. J., Chesa, P. G., Green, S. H., Mena, A. C., Old, L. J. (1988). Expression of human nerve growth factor receptor on cells derived from all three germ layers. Exp Cell Res. 174:533-539.

Timmusk ,T., Palm, K,. Metsis, M., Reintam, T., Paalme, V., Saarma, M., and Persson, H. (1993a). Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10: 475-89

Timmusk, T., Belluardo, N., Metsis, M., and Persson, H. (1993b). Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. Eur J Neurosci. 6:605-613.

To, C. T., and Tsao, M. S. (1998). The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers (Review). *Oncol. Rep.* 5, 1013-1024.

Trupp, M., Raynosche, k. C., Belluardo, N., and Ibanez, C. F. (1998). Multiple GPI-Anchored Receptors Control GDNF-Dependent and Independent Activation of the c-Ret Receptor Tyrosine Kinase.Mol Cell Neurosci. 11:47-63.

Tsoulfas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza, R. J., Rosenthal, A., Nikolics, K. and Parada, L. F. (1993). The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. *Neuron* 10: 975-990.

Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P. C., and Croce, C. M. (1985). Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature. 315:340-343

Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. (1995). Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatterfactor. Nature *373*: 702-705.

Ure, D. R., Campenot, R. B., and Acheson, A. (1992). Cholinergic

differentiation of rat sympathetic neurons in culture: effects of factors applied to distal neurites. Dev Biol. 154:388-395

Ullrich, A., Gray, A., Berman, C. and Dull, T. J. (1983). Human beta-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* 303: 821-825.

Valenzuela, D. M., Maisonpierre, P. C., Glass, D. J., Rojas, E., Nunez, L., Kong, Y., Gies, D. R., Stitt, T. N., Ip, N. Y. and Yancopoulos, G. D. (1993). Alternative forms of rat TrkC with different functional capabilities. *Neuron* 10: 963-974.

Vantini, G., Schiavo, N., Di Martino, A., Polato, P., Triban, C., Callegaro, L., Toffano, G., and Leon, A. (1989). Evidence for a physiological role of nerve growth factor in the central nervous system of neonatal rats. Neuron. 3: 267-273.

Varon, S., Nomura, J., and Shooter, E . M. (1968). Reversible dissociation of the mouse nerve growth factor protein into different subunits. Biochemistry. 7: 1296-1303.

Veis, D. J. , Sorenson, C. M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75:229-240.

Vekrellis, K., McCarthy, M. J., Watson, A., Whitfield, J., Rubin, L. L., and Ham, J. (1997). Bax promotes neuronal cell death and is downregulated during the development of the nervous system. Development. 124:1239-1249

Ventimiglia, R., Mather, P. E., Jones, B. E., and Lindsay, R. M. (1995). The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons in vitro. Eur J Neurosci. 7:213-222.

Verdi, J. M., Birren, S. J., Ibanez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V. and Anderson, D. J. (1994). p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* 12: 733-745.

Wall, P.D. (1995). Independent mechanisms converge on pain. *Nature Med.* 1: 740-741.

Wang, H. G., Miyashita, T., Takayama, S., Sato, T., Torigoe, T., Krajewski, S., Tanaka, S., Hovey, L 3rd., Troppmair, J., and Rapp, U. R. (1994a). Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. Oncogene. 9:2751-2756.

Wang, H. G., Millan, J. A., Cox, A. D., Der, C. J., Rapp, U. R., Beck, T., Zha, H., and Reed, J. C. (1995). R-Ras promotes apoptosis caused by growth factor deprivation via a Bcl-2 suppressible mechanism. J Cell Biol. 129:1103-1114.

Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996). BID: a novel BH3 domain-only death agonist. Genes Dev. 10: 2859-2869.

Wang, L., Muira, M., Bergeron, L., Zhu, H., and Yaun, J. (1994b). Ich-1 an ICE/ced-3 related gene, encodes both positive and negative regulators of programmed cell death. Cell. 78: 739-750.

Wang, S., Seldon, C., Farnaud, S., Calnan, D., and Hodgson, H. J. F. (1994c). Hepatocyte Growth Factor (HGF/SF) is expressed in human epithelial cells during embryonic development; studies by in-situ hybridisation and Northern blot analysis. J. Anat. 185: 543-551.

Weidner, K. M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. (1990). Scatter Factor: Molecular characteristics and effect on the invasiveness of epithelial cells. J. Cell. Biol. 111: 2097-2108.

Weidner, K. M., Arakaki, N., Hartmann, G., Vandekerckhove,J., Weingart, S., Daikuhara, Y., and Birchmeier, W. (1991). Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc. Natl. Acad. Sci. USA, 88:7001-7005.

Weidner, K. M., Di-cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996). Interaction between Gab1 and the c-met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature 384: 173-176.

Weidner, M. K., Sachs, M., and Birchmeier, W. (1993). The MET receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of Scatter Factor/ Hepatocyte Growth Factor in epithelial cells. J. Cell. Biol. 121: 145-154.

Weston, J. A. (1962). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. Dev. Biol. 6: 279-310.

White, F.A., Silos-Santiago, I., Molliver, D. C., Nishimura, M., Phillips, H., Barbacid, M., and Snider, W.D. (1996). Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. *J. Neurosci.* **16**: 4662-4672.

Whittemore, S. R., Friedman, P. L., Larhammar, D., Persson, H., Gonzalez, C. M. and Holets, V. R. (1988). Rat beta-nerve growth factor sequence and site of synthesis in the adult hippocampus. *J. Neurosci. Res.* 20: 403-410.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., and Olson, L. (1997). Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs.J Neurosci 1997 Nov 1;17(21):8506-8519

Wilkinson, G. A, Fariñas, I., Backus, C., Yoshida, C. K., Reichardt, L. F. (1996). Neurotrophin-3 is a survival factor in vivo for early mouse trigeminal neurons. *J Neurosci* . 16: 7661-7669.

Williams, G. T. (1991). Programmed cell death: apoptosis and oncogenesis. Cell.. 65:1097-1098.

Williams, L. R., Manthorpe, M., Barbin, G., Nieto-Sampedro, M., Cotman, C. W. and Varon, S. (1984). High ciliary neuronotrophic specific activity in rat peripheral nerve. *Int. J. Dev. Neurosci.* 2: 177-180.

Williams, L. R., Varon, S., Peterson, G. M., Wictorin, K., Fischer, W., Bjorklund, A., and Gage, F. H. (1986). Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc. Natl. Acad. Sci. U S A. 23:9231-9250

Wollert, K. C., Taga, T., Saito, M., Narazaki, M., Kishimoto, T., Glembotski, C. C., Vernallis, A. B., Heath, J. K., Pennica, D., Wood, W. I., and Chien, K. R. (1996). Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. J Biol Chem. 271:9535-9545

Wong, V., Glass, D.J., Arriaga, R., Yancopoulos, G.D., Lindsay, R.M., and Conn, G. (1997). Hepatocyte growth factor promotes motor neuron survival and synergizes with ciliary neurotrophic factor. J. Biol. Chem. *272*: 5187-5191.

Woolf, A. S., Kolatsi-Joannou, M., Hardman, P., Andermarcher, E., Moorby, C., Fine, L. G., Jat, P. S. Noble, M. N., and Gherardi, E. (1995). Roles of Hepatocyte Growth Factor/ Scatter Factor and the Met receptor in the early development of the metanephros. J . Cell. Biol. 128: 171-184.

Worby, C. A., Vega, Q. C., Chao, H. H., Seasholtz, A. F., Thompson, R. C., and Dixon, J. E. (1998). Identification and characterization of GFRalpha-3, a novel Co-receptor belonging to the glial cell line-derived neurotrophic receptor family.J Biol Chem. 273:3502-3508

Wright, D. E., Zhou, L., Kucera, J., and Snider, W. D. (1997). Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. Neuron. 19:503-517

Wright, E. M., Vogel, K. S. and Davies, A. M. (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron* 9: 139-150.

Wright, E. M., Vogel, K. S., and Davies, A. M. (1992). Neurotrophic Factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9, 139-150.

Wyatt, S., and Davies, A. M. (1995). Regulation of nerve growth factor receptor gene expression in sympathetic neurons during development. J. Cell Biol. *130*: 1435-1446.

Wyatt, S., Pinon, L. G., Ernfors, P., and Davies, A. M. (1997). Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos.EMBO J. 16:3115-23

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. Int Rev Cytol. 68:251-306.

Xue, D., and Horvitz, H. R. (1995). Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in Baculovirus p35 protein. Nature, 377: 248-251.

Xue,D., Shaham , S. and Horvitz, H. R. (1996). The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substratespecificities similar to those of the human CPP32 protease.Genes Dev. 9:1073-1083

Yamada, T., Tsubouchi, H., Daikuhara, Y., Prat, M., Comoglio, P. M. McGeer., and McGeer, E.G. (1994). Immunohistochemistry with antibodies to hepatocyte growth factor and its receptor protein (c-Met) in human brain tissue. Brain Res 637: 308-312.

Yamagata, T., Muroya, K., Mukasa, T., Igarashi, H., Momoi, M., Tsukahara, T., Arahata, K., Kumagai, H., and Momoi, T. (1995). Hepatocyte Growth Factor specifically expressed in microglia activated Ras in the neurons, similar to the action of neurotrophic factors. Biochem. Biophys. Res. Comm. 1: 231-237.

Yamamoto, Y., Livet, J., Vejsada, R., Pollock, R.A., Arce, V., deLapeyriere, O., Kato, A.C., and Henderson, C.E. 1997. Hepatocyte growth factor (HGF/SF) is an essential muscle-derived survival factor for a subpopulation of embryonic motoneurons. Development *124*: 2903-2913.

Yan, H. and Chao, M. V. (1991). Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. *J Biol Chem* 266: 12099-12104.

Yan, Q., Elliott, J. and Snider, W. D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* 360: 753-755.

Yanagita, K., Matsumoto, K., Sekiguchi, K., Ishibashi, H., Niho, y., and Nakamura, T. (1993). Hepatocyte Growth Factor may act as a pulmotrophic factor on lung regeneration after acute lung injury. J. Biochem Chem. 268: 21212-21217.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones D. P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 275:1129-1132.

Yang, X. M., Toma, J. G., Bamji, S. X., Belliveau, D. J., Kohn, J., Yasuda, T., Sobue, G., Ito, T., Mitsuma, T., and Takahashi, A. (1990). Nerve growth factor enhances neurite arborization of adult sensory neurons; a study in single-cell culture.Brain Res. 254:54-63

Yaun, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian Interleukin-1β-converting enzyme.Cell. 75: 641-652.

Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369: 321-323.

Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H., and Lindholm, D. (1990). Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J. 9:3545-3550.

Zafra, F., Castren, E., Thoenen, H., and Lindholm, D. (1991). Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. Proc. Natl. Acad. Sci. U S A. 88:10037-10041.

Zarnegar, R., and Michalopoulos, G. K. (1989). Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. Cancer Res. 49: 3314-3320.

Zarnegar, R., DeFrances, M. C., Oliver, L., and Michalopoulos, G. K. (1990). Identification and partial characterization of receptor binding sites for HGF on rat hepatocytes. Biochem. Biophys. Res. Comm. 173: 1179-1185.

Zarnegar, R., and Michalopoulos, G. K. (1995). The many faces of Hepatocyte Growth Factor: From Hepatopoiesis to Hematopoiesis. J . Cell Biol. 129: 1177-1180.

Zhang, K. L., Westberg, J. A., Holtta, E., and Andersson, L. C. (1996). Bcl-2 regulates neural differentiation. Proc. Natl. Acad. Sci. USA. 93: 4504-4508.

Zheng, J. L., Stewart, R. R. and Gao, W. Q. (1995a). Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin induced neurotoxicity. *J. Neurosci.* 15: 5079-5087.

Zheng, J. L., Stewart, R. R. and Gao, W. Q. (1995b). Neurotrophin-4/5, brain derived neurotrophic factor, and neurtrophin-3 promote survival of cultured vestibular ganglion neurons and protect them against neurotoxicity of ototoxins. J. neurobiol. 28: 330-340.

Zhong, L. T., Sarafian, T., Kane, D. J., Charles, A. C., Mah, S. P., Edwards, R. H., Bredesen, D. E. (1993b). bcl-2 inhibits death of central neural cells induced by multiple Agents. Proc. Natl. Acad. Sci. U S A. 90:4533-4537.

Zhou, X., and Rush, R. A. (1995). Sympathetic neurons in neonatal rats require endogenous neurotrophin-3 for survival. J. Neurosci. *15*: 6521-6530.

Zhu, H., Naujoka, M. A., Fixman, E. D., Torossian, K., and Park, M. (1994). Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. J. Biol. Chem. 269: 29943-29948.